Probing the IgE-Binding Repertoire of Self-Antigens in Atopic Eczema

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Publications

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2 Summary

Atopic eczema is the most common chronic inflammatory skin disease, affecting 10-20% of children and 1-3% of adults in industrialized countries. Various genetic, environmental, and physiological factors act together in the multifactorial pathogenesis of atopic eczema by activating well-known immunological and inflammatory pathways. In addition, IgE-mediated reactivity against human proteins, so called autoreactivity, has been postulated as a further pathogenic mechanism. Already in 1941 it was described that human skin dander is able to trigger immediate type skin reactions in patients with severe atopic eczema, suggesting that endogenous proteins may serve as targets for IgE autoantibodies. However, only the application of modern molecular biology methods led to the elucidation of the biochemical nature of these self-antigens. Although several IgE-binding self-antigens associated with atopic eczema are described in deep detail, the whole repertoire of these proteins and their role in the pathogenesis of the disease are still largely unknown. Therefore, the aim of this thesis was the identification of new IgE-binding self-antigens associated with atopic eczema and the characterization of their immunological properties and their expression in the skin.

To identify new IgE-binding self-antigens, a human cDNA library displayed on phage surface was screened with solid-phase immobilized serum IgE of patients suffering from atopic eczema. 140 complete and partial sequences encoding putative IgE-binding self-antigens were identified. The presence of nine out of twelve already described self-antigens among the enriched clones indicated a high specificity of the screening system used. Five putative self-antigens namely actin-alpha, tubulin-alpha, eukaryotic initiation factor for translation 6, HLA-DR-alpha, and RP1 were randomly selected for cloning, expression as His_6-tagged fusion proteins in *Escherichia coli*, and purification by Ni^{2+}-chelate affinity chromatography. Western blot as well as ELISA experiments confirmed that the recombinant self-antigens bind IgE of sera of atopic eczema patients while healthy controls had no detectable levels of self-antigen-specific IgE. Moreover, the recombinant self-antigens cross-linked receptor bound IgE on the surface of basophils as demonstrated by the upregulation of CD63 and CD203c and induced the proliferation of peripheral blood mononuclear cells of sensitized patients. Interestingly, immunohistochemistry staining of skin biopsies demonstrated that tubulin-α and HLA-DR-α are upregulated in acute and chronic atopic eczema lesions compared to unaffected skin from the same patient or healthy controls.

In summary it can be ascertained that the spectrum of IgE-binding self-antigens involved in the pathogenesis of AE is much broader than initially expected. Many human proteins might serve as IgE-binding self-antigens in atopic eczema and most atopic eczema patients have autoreactive IgE-antibodies in serum. Self-antigens are supposed to exacerbate the chronic inflammation in the skin by activating the humoral and cellular immunity. These results are of considerable significance for the management of atopic eczema and the definition of new therapeutic strategies.

3 Zusammenfassung

Mit einer Prävalenz von 10-20% unter Kindern und 1-3% unter Erwachsenen ist das atopische Ekzem die häufigste chronisch-inflammatorische Hauterkrankung in den Industrieländern. Zahlreiche genetische, physiologische und umweltbedingte Faktoren wirken bei der Pathogenese zusammen und aktivieren immunologische und inflammatorische Signalwege. Zudem wurden IgE-vermittelte Reaktionen gegen humane Proteine als weitere Ursache für die Entstehung des atopischen Ekzems vorgeschlagen.

Schon 1941 wurde beschrieben, dass menschliche Hautschuppen bei Patienten mit schwerem atopischen Ekzem Typ I-Hautreaktionen auslösen können. Diese Beobachtungen ließen vermuten, dass auch endogene Proteine IgE-Antikörper binden können. Doch erst mit der Anwendung von modernen Methoden der Molekularbiologie ließ sich die biochemische Natur dieser Autoantigen aufklären. Obwohl mehrere IgE-bindende Autoantigene im Zusammenhang mit dem atopischen Ekzem detailliert beschrieben sind, ist das gesamte Spektrum dieser Proteine und deren Rolle in der Pathogenese des atopischen Ekzems noch weitestgehend unbekannt.

Das Ziel dieser Dissertation war daher die Bestimmung neuer IgE-bindender Autoantigene, die Charakterisierung ihrer immunologischen Eigenschaften sowie die Untersuchung ihrer Expression in der Haut. Um neue IgE-bindende Autoantigene zu bestimmen, wurde eine humane Phage-Display Bibliothek mit immobilisierten IgE-Antikörpern aus dem Serum von Patienten mit atopischem Ekzem gescreent. Dabei wurden 140 vollständige und partielle DNA-Sequenzen gefunden, die für potentielle IgE-bindende Autoantigene kodieren. Da unter diesen auch neun von zwölf Autoantigenen präsent waren, welche bereits im Zusammenhang mit dem atopischen Ekzem beschriebenen sind, scheint das verwendete Screening-System hoch spezifisch zu sein. Fünf potentielle Autoantigene, nämlich Aktin-α, Tubulin-α, eukaryotischer Translationsinitiationsfaktor 6, HLA-DR-α und RP1 wurden zufällig ausgewählt, kloniert, als His_6-tagged Fusionsproteine in *Escherichia coli* exprimiert und mittels Affinitätschromatographie gereinigt. Western blot- und ELISA-Experimente bestätigten, dass die rekombinanten Proteine IgE im Serum von Patienten mit atopischem Ekzem binden, während im Serum von gesunden Kontrollen keine spezifischen IgE-Antikörper nachzuweisen waren. Des Weiteren kreuzvernetzen die rekombinanten Autoantigene Rezeptor-gebundenes IgE an der Oberfläche von Basophilen, wie die Hochregulierung von CD63 und CD203c zeigte, und induzierten die Proliferation von mononukleären Zellen des peripheren Blutes von sensibilisierten Patienten. Mittels Immunohistochemie

wurde gezeigt, dass Tubulin-α und HLA-DR-α in Biopsien von akuten und chronischen Läsionen im Vergleich zu unbetroffener Haut der selben Patienten oder Haut von gesunden Kontrollen hochreguliert sind.

Zusammenfassend kann angenommen werden, dass das Spektrum von IgE-bindenden Autoantigenen, die im Zusammenhang mit dem atopischem Ekzem stehen, deutlich breiter ist als ursprünglich angenommen. Viele humane Proteine können möglicherweise als IgE-bindende Autoantigene dienen und die meisten Patienten mit atopischem Ekzem haben autoreaktive IgE-Antikörper. Durch die Aktivierung der humoralen- und zellulären Immunität verschlimmern diese Autoantigene scheinbar die chronische Entzündung in der Haut. Diese Ergebnisse sind von beachtlicher Bedeutung bei der Behandlung des atopischen Ekzems und der Definition von neuen therapeutischen Strategien.

4 Abbreviations

3D	three-dimensional
A. fumigatus	*Aspergillus fumigatus*
Ab	antibody
ABPA	allergic bronchopulmonary aspergillosis
ADCC	antibody-dependent cell-mediated cytotoxicity
AE	atopic eczema
Ag	antigen
AICD	activation-induced cell death
AIRE	autoimmune regulator
BCR	B cell receptor
CD	cluster of differentiation
cTEC	cortical thymic epithelial cell
CTLA-4	cytotoxic T lymphocyte antigen 4
DC	dendritic cell
E. coli	*Escherichia coli*
EAE	experimental autoimmune encephalitis
eIF6	eukaryotic initiation factor for translation 6
ELISA	enzyme-linked immunosorbent assay
FcR	receptor for constant region of an antibody
FoxP3	forkhead box P3
GM-CSF	granulocyte macrophage growth factor
GMP	good manufacturing practice
HLA	human leukocyte antigen
IDEC	inflammatory dendritic epidermal cell
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IMAC	immobilized metal affinity chromatography
LAG-3	lymphocyte activation gene-3
LFA-1	lymphocyte function-associated antigen-1
LPS	lipopolysaccharide
M. sympodialis	*Malassezia sympodialis*
MBP	myelin basic protein

MHC	major histocompatibility complex
MnSOD	manganese superoxide dismutase
mTECs	medullary thymic epithelial cells
PAMP	pathogen-associated molecular patterns
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PD-1	programmed death 1
S. aureus	*Staphylococcus aureus*
SIT	specific immunotherapy
STAT	signal-transducing activators of transcription
TCR	T cell receptor
TGF	transforming growth factor
Th cell	T helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
Tr1	type 1 regulatory T cell
Treg cell	regulatory T cell
TSA	tissue specific antigen
TSLP	thymic stromal lymphopoietin

5 Introduction

5.1 Historical background of allergy

Today allergic diseases are the most common chronic illnesses in childhood and up to 30% of adults in Western countries are suffering from allergic rhinitis, allergic asthma, or atopic eczema (AE) (1). Allergy, however, is not a new disease but well recognized since antiquity: The earliest report of an allergic reaction dates back to 3500 BC when King Menses of Egypt was killed by the sting of a wasp. The Greek physician Hippocrates (460-357 BC) first described clinical symptoms comparable to asthma and AE. Around 50 BC the Roman poet and philosopher Titus Lucretius Carus (97-55 BC) observed exaggerated responses to commonly occurring substances and wrote in *De Rerum Natura* (On the Nature of Things) “what is food for some may be fierce poisons for others” (2).

The modern era of allergology started in the 1800s with the description of hay fever by John Bostock (3), followed by the identification of pollen as causative agents for allergic reactions by Charles Blackely in 1873 (4). In 1906 the Austrian pediatrician Clemens von Pirquet first introduced the word “allergy” to describe the symptoms, which some diphtheria patients developed after treatment with a horse serum antitoxin (5). The word derives from the Greek terms “allos” (other) and “ergon” (reaction) and expresses “the altered capacity of the body to react to a foreign substance”. Scientific investigations on the immunological basis of allergic reactions were initiated by the classic experiment of Carl W. Prausnitz and Hans Küstner in 1921: Intradermal injection of serum from a sensitized patient to a normal person leads to a local weal and flare response after challenging with the sensitizing allergen (6). The identification of immunoglobulin (Ig) E as the responsible factor transferring local hypersensitivity (7, 8) marks a major breakthrough in understanding the mechanisms of allergy.

5.2 Hypersensitivity reactions and IgE-mediated allergy

In 1968 IgE was described as a novel immunoglobulin subclass (9) and found to be the triggering factor for allergic diseases (7). Beside its postulated effects in the protective immunity against helminth infections not much is known about the physiological role of

IgE antibodies. In contrast, the pathophysiology of IgE-mediated diseases has been studied in detail.

During primary sensitization antigen-presenting cells, mainly dendritic cells (DCs), take up and process the antigen and present the resulting peptides via MHC class II molecules to naïve T cells (Figure 1). Upon activation naïve T cells differentiate into allergen-specific T effector cells. IFN-γ and IL-12 drive the differentiation of naïve $CD4^+$ T cells into T helper (Th) 1 cells, whereas exposure to IL-4, IL-5, IL-9, and IL-13 favors the differentiation of Th2 cells. They are characterized by the expression of the transcription factor GATA-3 and the production of IL-4, IL-5, IL-9, and IL-13 and constitute the prominent T cell subset in allergic inflammation (Figure 2). Mature Th2 cells activate allergen-specific B cells that differentiate into antibody secreting plasma cells. IL-4 and IL-13 induce via the transcriptional regulator STAT6 an isotype switch to IgE expression. Secreted IgE binds to its high affinity receptor FcεRI on the surface of mast cells, basophils, and activated eosinophils. Cross-linking of receptor-bound IgE by specific antigens leads to degranulation of the effector cells, resulting in the immediate release of various preformed or newly synthesized inflammatory mediators (10).

Mast cells reside in the connective tissue, most abundantly in the submucosal tissue and the dermis. Within seconds upon stimulation they release histamine, a vasoactive amine that increases local blood flow and vascular permeability. Enzymes, such as mast cell chymase, tryptase, and cathepsin G activate matrix metalloproteinases, which break down tissue matrix proteins leading to tissue destruction. TNF-α activates endothelial cells that express adhesion molecules to promote the influx of leukocytes and lymphocytes into the tissue. Lipid mediators such as prostaglandin D2 or leukotrienes cause smooth muscle contraction, increased vascular permeability, and mucus secretion. Furthermore, mast cells secrete cytokines such as IL-4 and IL-13 that amplify the allergic response by promoting the differentiation of Th2 cells and IgE class switch. IL-3, IL-5, and GM-CSF induce eosinophil production in the bone marrow. After release into circulation they are attracted to the site of inflammation by eotaxins, produced by leukocytes. By releasing toxic granule proteins and free radicals eosinophils are able to kill microorganisms but may also cause tissue damage in allergic inflammation (10).

The recruitment, activation, and effector functions of mast cell, basophils, and eosinophils closely correlate. IL-3, IL-5, GM-CSF induce the growth of both basophils and eosinophils. Their maturation is, however, reciprocally controlled. Basophils, eosinophils, and mast cells are recruited by the same group of CC chemokines, including CCL7, CCL13, and CCL5. Prostaglandin D2, which is released by mast cells, recruits eosinophils, basophils, and Th2 cells. The degranulation of mast cells and basophils can be induced by major basic protein, which is released by eosinophils (10).

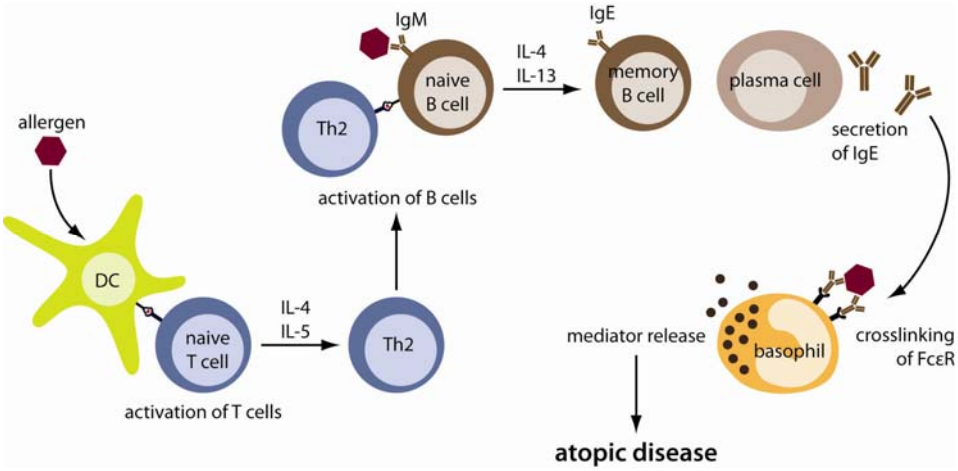

Figure 1. Type I hypersensitivity reaction. Antigen-presentation by DCs leads to the activation and differentiation of Th2 cells that in turn activate naïve B cells. B cells turn into IgE-expressing memory B cells and IgE-secreting plasma cells. Secreted IgE binds to FcεRI on basophils and mast cells. Specific allergens cross-link receptor-bound IgE, leading to the release of inflammatory mediators such as histamine and leukotriens and the symptoms of allergic diseases.

Clinically the allergic response is divided into an immediate reaction (type I hypersensitivity reaction) and a late phase reaction (Table 1). The immediate response is due to the release of preformed or rapidly synthesized molecules such as histamine or prostaglandins, respectively. After 7-9 hours the late-phase-reaction develops in about 50% of patients with an immediate reaction. Newly synthesized lipid mediators, cytokines, and chemokines recruit additional leukocytes and Th2 cells to the site of inflammation, resulting in edema and tissue remodeling by increased numbers and enhanced growth of smooth muscle cells.

In contrast to type I hypersensitivity reactions, type II and III hypersensitivity reactions are mediated by IgG antibodies. Binding of cell surface and matrix-associated allergens (type II) or soluble antigens (type III) activates complement-meditated and phagocytic effector mechanisms, leading to drug allergies or serum sickness and systemic lupus erythematosus, respectively.

Type IV hypersensitivity reactions are characteristic for long lasting allergic inflammations that reach a chronic stage and lead to severe long-term illness, such as chronic allergic asthma or AE. These type IV hypersensitivity reactions are mediated by both Th1 and Th2 cells and their specific cytokines IFN-γ and IL-4, IL-5, and IL-13, respectively (10).

	Type I	Type II	Type III	Type IV
Immune mechanisms	IgE-mediated	IgG-mediated	immune complex-mediated, IgG-mediated	T cell-mediated
Antigen	soluble	cell- or matrix-associated	soluble	soluble or cell-associated
Effector mechanism	cross-linking of FcεR-bound IgE on mast cells and basophils leads to their degranulation	cell- or matrix-bound Ag induce opsonization and phagocytosis of cells, the activation of the complement cascade, and antibody-dependent cell-mediated cytotoxicity (ADCC)	Ag-Ab complexes deposited in various tissues induce activation of the complement cascade and infiltration of FcR^+ cells	sensitized Th1 cells release cytokines that activate macrophages, eosinophils, or CTLs, leading to tissue injury or direct cell killing
Symptoms	allergic rhinitis, acute allergic asthma, acute atopic eczema, systemic anaphylaxis	drug allergies, blood transfusion	serum sickness, Arthus reaction, systemic lupus erythematosus	contact dermatitis, chronic allergic asthma, chronic atopic eczema, graft rejection
Time to develop	minutes	hours	hours to weeks	two to three days

Table 1. Hypersensitivity reactions. In 1963 Coombs and Gell classified hypersensitivity reactions into four types according to the immunological mechanisms, cells, and mediator molecules causing tissue damage (11). (Adapted from Abbas AK et al. (12) and Murphy KM et al. (10))

5.3 Immunological tolerance

The mammalian immune system possesses the remarkable capacity to defend against numerous invading pathogens while maintaining immunological unresponsiveness to self-antigens. By suppressing immune responses deleterious to the host, immunological tolerance is crucial for a healthy immune response and the prevention of autoimmunity or allergic diseases. Ensuring self-tolerance within the lymphocyte repertoire is exerted at two levels: Central tolerance refers to the deletion of developing autoreactive B and T cells that bind self-antigens in the bone marrow or thymus, respectively. Despite stringent selection, not all lymphocytes capable of recognizing self-antigens are eliminated during their development. Those cells that evade central tolerance are controlled by peripheral regulatory mechanisms including ignorance, anergy, phenotypic skewing, tolerogenic DCs, and regulatory T (Treg) cells.

5.3.1 Central tolerance

In humans a T cell repertoire of approximately 25 million specificities has been described (13). However, during the maturation of thymocyte progenitors in the thymus random gene rearrangements also lead to useless or even harmful receptor specificities. Positive selection is the first step in central tolerance and leads to the enrichment of MHC-restricted T cell progenitors. Thereby double-positive T cell progenitors interact with cortical thymic epithelial cells (cTECs) in the thymus cortex. Binding of the lymphocytes' T cell receptor (TCR) to self-peptide-MHC complexes on cTEC allows the further differentiation of T cell progenitors while progenitors with no or low affinity die by neglect (14). Surviving double-positive progenitors express the chemokine receptor CCR7 and migrate towards the thymic medulla where negative selection takes place (15, 16).
Negative selection is crucial to eliminate potential autoreactive cells generated during positive selection. Therefore medullary thymic epithelial cells (mTECs) that express costimulatory molecules and peripheral tissue specific antigens (TSAs) under the control of the transcriptional regulator AIRE, interact with T cell progenitors during the transition from double-positive to single-positive stage (14). Beside mTECs also DCs present in the medulla are able to induce negative selection by cross-presenting TSAs generated by mTECS and the expression of costimulatory molecules such as CD40, CD80 and CD86 (17). High affinity binding of TCR to self-antigens presented by mTECs or DCs induces clonal deletion or anergy of both $CD4^+$ and $CD8^+$ T cells. Very high affinity for self-peptides is supposed to induce the differentiation of natural Treg cells that regulate immune responses in the periphery and maintain self-tolerance (18, 19).

Similar mechanisms occur in the bone marrow during development of immature B cells: Binding of IgM to multivalent self-antigens, for example to ubiquitous surface molecules such as MHC, induces clonal deletion of immature B cells. These cells either undergo apoptosis or receptor editing by secondary gene rearrangements of the light chain loci. The interaction of progenitor B cells with soluble self-antigens, which are able to cross-link the B cell receptor (BCR) renders these cells anergic to the antigen. They migrate to the periphery, but do not respond to the antigen and are rapidly lost in competition with other B cells. Immature B cells that bind monovalent antigens or self-antigens with very low affinity, which are not able to cross-link the BCR do not receive a negative signal and develop normally. However, those cells are clonally ignorant since their specific ligand is present but not able to activate them (20).

5.3.2 Peripheral tolerance

Although central tolerance is a crucial mechanism to avoid the development of autoreactivity, not all self-reactive lymphocytes are deleted in the thymus or bone marrow. Some TSAs are represented on the mRNA level, but not necessarily on the protein level in the thymus. Moreover, extremely stringent negative selection would narrow the repertoire of available TCR specificities that respond to foreign pathogens (21). Therefore, some T cells with a degree of self-reactivity escape deletion and exit the thymus. Indeed, healthy individuals have autoreactive T cells with low-affinity TCR for self-antigens in the periphery (22, 23). These autoreactive cells are controlled by T cell intrinsic mechanisms acting directly on the self-reactive T cells and by T cell extrinsic mechanisms including tolerogenic DCs and Treg cells.

Four T cell intrinsic mechanisms to control self-reactive T cells have been described: ignorance, anergy, phenotype skewing, and apoptosis. Ignorance occurs if the antigen is anatomically separated and not accessible for effector T cells (24) or does not reach the threshold required to trigger a T cell response (25). Anergy refers to a state of unresponsiveness to a specific antigen and was first reported as the results of TCR-ligation in the absence of co-stimulation *in vitro* (26). Later studies demonstrated that activation of inhibitory receptors such as CTLA-4 and PD-1 by CD80/86 and PDL1/2, respectively, plays a role in the induction of anergy (27-29). Phenotype skewing describes the observation that self-reactive T cells undergo full activation, but develop a non-pathogenic phenotype in terms of cytokine and chemokine receptor expression. For example Th2 cytokines are shown to downregulate autoimmunity and altered lymphocyte trafficking may prevent the migration of autoreactive T cells to B cell areas in lymphoid organs (30, 31). Finally, self-reactive T and B cells can die by activation-induced cell death (AICD) (32). Thereby contact with self-antigens leads to the upregulation of Fas-ligand and apoptosis by signaling through the death receptor Fas (CD95). AICD is not supposed to eliminate autoreactive T cell precursors completely, but is supposed to reduce their numbers to a level where anergy can be efficiently induced.

The T cell extrinsic mechanisms controlling autoreactivity include tolerogenic DCs and Treg cells. Whether any immature DC is able to induce tolerogenic functions or whether this task is mediated by a specific DC subset is not known. Following exposure to pathogen-associated molecular patterns (PAMPs) or intracellular products released during cellular damage, immature DCs modify their antigen-processing capacity and their surface phenotype. The high expression of costimulatory molecules such as CD80/86 and CD40 allows the activation of T cells and the initiation of an immune response (10).

Consequently, the absence of PAMPs and the low expression of costimulatory molecules by DCs leads to anergy or clonal deletion rather than T cell activation (33, 34). Moreover, it is assumed that immature or tolerogenic DCs maintain tolerance by inducing Treg cells (35), a distinct subset of T cells with regulatory functions (see chapter 5.3.3).

5.3.3 Regulatory T cells

Treg cells are able to inhibit autoreactivity as well as allergic responses and are important in maintaining allergen-specific peripheral tolerance (36). Different subsets of Treg cells have been described, including induced Tr1 cells (37), Th3 cells (38), and naturally occurring $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells (39, 40). The latter develop in the thymus, express the transcription factor forkhead box P3 (FoxP3), and display a T cell receptor repertoire that is specific for self-antigens. In contrast to naturally occurring Treg cells, the generation of Tr1 and Th3 cells can be induced by IL-10 and transforming growth factor-β (TGF-β) in peripheral tissues (Figure 2) (41). Moreover, high dose of allergen exposure induces a rapid switch and expansion of IL-10-producing Tr1 cells in non-allergic individuals (42).

The development and function of Treg cells are regulated by an IL-2-mediated negative feedback loop. FoxP3 together with other transcription factors and cofactors represses the transcription of IL-2 in Treg cells and renders them highly dependent on exogenous IL-2. Antigenic stimulation of T effector cells induces the secretion of IL-2, which binds to its high-affinity receptor on Treg cells and in turn contributes to the maintenance, expansion, and activation of natural Treg cells and facilitates the TGF-β dependent differentiation of induced Treg cells (43).

Beside $CD4^+$ and $CD8^+$ T cells, Treg cells also suppress the activities of natural killer cells, natural killer T cells, B cells, macrophages, osteoclasts, and dendritic cells by various synergistic suppressive mechanisms (43, 44). Upon antigenic stimulation in the thymus Treg cells are recruited via chemokines to DCs. The high expression of adhesion molecules such as LFA-1 by Treg cells allows long-lasting contact between Treg cells and DCs, leading to out-competition of naïve T cells and prevention of their activation. In addition, Treg cells are able to modulate the maturation and function of DCs, which are required for the activation of effector T cells. LAG-3 on the surface of Treg cells binds to MHC class II molecules on immature DC and blocks their maturation. Upon interaction between CTLA4 and CD80 or CD86 DC secrete indoleamine 2,3-dioxygenase (IDO), an immunosuppressive mediator, which induces the production of pro-apoptotic molecules in effector T cells (45). Treg cells also kill or inactivate effector T cells directly by secreting inhibitory cytokines such as IL-10, TGF-β, and IL-35 that suppress the generation and activation of effector cells (46, 47). The release of granzyme A and B together with

perforin mediates cytolysis of target cells (48). In addition Treg cells are supposed to elicit metabolic disruption in effector cells. High-level expression of CD25 (IL-2 receptor α) by Treg cells leads to the local consumption of IL-2, resulting in cytokine-deprivation and apoptosis of dividing effector T cells (49). Finally, released adenosine and cAMP are able to inhibit effector T cell function through binding of the adenosine receptor 2A on the cell surface or by direct transfer into effector cells via gap junctions, respectively (50, 51).
The concentration of antigenic peptides required to activate Treg cells is estimated to be 10- to 100-fold lower than the concentration needed to activate naïve T cells (52). Thus, Treg cells may prevent autoimmunity induced by molecular mimicry because Treg cells are more easily activated by suboptimal T cell receptor ligand concentrations than cross-reactive T cells (53).

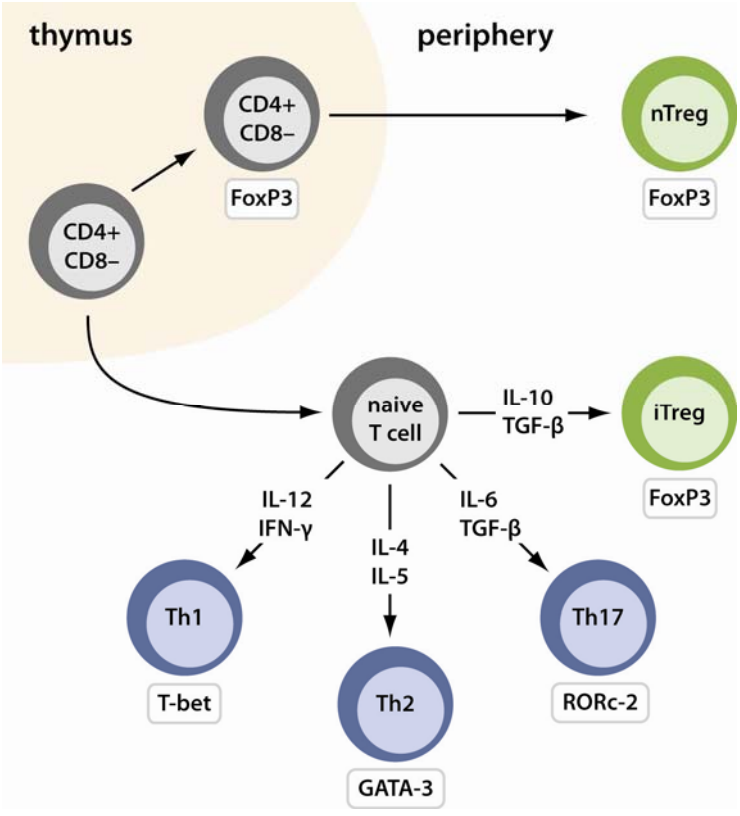

Figure 2. Differentiation of naïve CD4$^+$ T cells into Treg cells or effector T cells. CD4$^+$ T cells differentiate into FoxP3$^+$ natural Treg cells (nTreg) in the thymus or migrate into the periphery where they differentiate into induced Treg cells (iTreg), Th1, Th2, or Th17 cells, depending on the cytokines present. The transcription factors T-bet, GATA-3, RORc-2, and FoxP3 are required for the differentiation of naïve T cells into Th1, Th2, Th17, and Treg cells, respectively. (Adapted from Sakaguchi S et al. (43))

In healthy individuals Treg cells constitute the major T cell subset specific for environmental allergens, whereas in allergic patients IL-4 secreting Th2 cells predominate (54). Treg cells are supposed to prevent allergic symptoms by suppressing the generation and effector functions of T cells, eosinophils, mast cells and basophils. IL-10 and TGF-β inhibit the production of IgE but induce the secretion of non-inflammatory antibodies of the IgG4 and IgA isotype in B cells (55). Suppressed Th2 cells can neither provide cytokines such as IL-4 and IL-13, which induce class-switch to IgE, nor support the differentiation, the survival, and the activation of eosinophils, mast cells, and basophils by IL-3, IL-4, and IL-5. In addition, suppressed Th1 cells do not mediate tissue injury such as apoptosis of skin keratinocytes or bronchial epithelial cells.

5.4 Atopic diseases

The immune system is crucial for human health and survival by providing host defense against infectious agents. However, sometimes immune responses mount against antigens, which are not associated with pathogens, and lead to severe diseases: Immune responses against self-antigens result in autoimmunity characterized by chronic inflammation and tissue damage, whereas undesirable reactions against harmless environmental antigens such as pollens, animal dander, moulds, or food are the cause of allergic diseases.

Allergen-specific IgE antibodies are necessary, but not sufficient to develop allergic diseases. However, about 40% of individuals sensitized to inhalant allergens are asymptomatic (56). This finding demonstrates that beside environmental factors also genetic predisposition contributes to the development of allergic diseases. In so-called atopic individuals with elevated serum IgE levels and increased numbers of eosinophils, several susceptibility genes on different loci increase the risk of developing AE, allergic rhinitis, or allergic asthma.

Usually the onset of AE occurs during the first 6 months of life and frequently improves during childhood. However, around 40% of children suffering from AE undergo the atopic march and develop allergic rhinitis or allergic asthma, respectively (57).

5.4.1 Atopic eczema

AE is the most common inflammatory skin disorder affecting 10-20% of children and 1-3% of adults in industrialized countries (58, 59). It presents as a relapsing, highly pruritic inflammation during infancy but can also persist or start in adulthood. The pathogenesis of AE is multifactorial, based on a strong genetic predisposition and triggered by various

environmental and physiological factors, leading to skin barrier dysfunction and chronic inflammation (Figure 3).

80% of the AE patients suffer from the atopic form of the disease (previously called extrinsic AE), which is characterized by elevated levels of total serum IgE (>150 kU/l), accompanied with sensitizations to environmental allergens, allergic rhinitis, or allergic asthma. In contrast, patients with non-atopic eczema (intrinsic AE) show normal levels of total serum IgE but eczematous skin lesions, which are clinically and histologically indistinguishable from those observed in extrinsic atopic eczema (60, 61).

Figure 3. Clinical finding of AE. In infancy first eczematous lesions usually emerge on the cheeks, the forehead and the scalp. Scratching causes crusted erosions.

Immunologically the pathogenesis of extrinsic AE has a biphasic nature: It is initiated by a type I hypersensitivity reaction characteristic during the acute phase, followed by a characteristic type IV hypersensitivity reaction during the chronic phase of the disease (Figure 4). During the acute phase antigen-presenting Langerhans cells in the epidermis favor the differentiation of naïve T cells into Th2 cells secreting IL-4, IL-5, and IL-13, which induces an isotype switch to IgE expression in B cells. Chemokines that are expressed by endothelial cells and keratinocytes attract leukocytes to the site of inflammation. For instance, in acute AE increased serum levels of monocytes chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP-1β), eotaxin, IL-16, and RANTES are observed (62). High numbers of recruited mast cells secrete

histamine, which induces pruritus by binding to histamine H3 receptors on sensory nerves. Moreover, histamine acts as a leukocyte chemoattractant and upregulates the production of various inflammatory chemokines and cytokines such as IL-6, IL-8, and GM-CSF by keratinocytes via histamine H1 receptors (63). The migration of memory and effector T cells to the inflammed skin is mediated predominantly by cutaneous lymphocyte antigen (CLA) and the chemokines CCL27 and CCL18. Langerhans cells residing in the epidermis express FcεRI on their surface and aggregation of the receptor leads to the release of additional chemotactic factors such as IL-16, CCL22, and CCL17 and the recruitment of inflammatory dendritic epidermal cells (IDEC) (64). Moreover, the IL-7-like cytokine thymic stromal lymphopoietin (TSLP) released by epithelial cells further enhances the secretion of IL-16 from Langerhans cells and the recruitment of $CD4^+$ T cells, monocytes, and IDEC. In contrast to Langerhans cells, IDEC are found exclusively at inflammatory sites. Upon FcεRI cross-linking they secrete IL-12 and IL-18 and enhance the priming of naïve T cells into Th1 cells, which dominate the inflammatory response during the chronic phase of AE (Figure 2, Figure 4). In contrast to psoriatic lesions, low numbers of Th17 cells are found in AE lesions (65) and naturally occurring $CD4^+$ $CD25^+$ $FoxP3^+$ Treg cells are found to be absent from AE lesions (66).

By expressing membrane-bound and soluble Fas ligands (FasL) as well as IFN-γ Th1 cell induce keratinocyte apoptosis (67), leading to disruption of the skin barrier. Moreover, a slight, prolonged alkalinization from pH 5.0 to pH 5.5 in the skin of AE patients sustains the activity of serine proteases, which are involved in the proteolysis of corneodesmosomes and lipid processing enzymes (68). Degradation of sphingomyelinase results in decreased content of ceramides and an altered lipid layer (69). In addition impaired expression of cornified envelope proteins such as filaggrin and keratins promotes the skin barrier dysfunction, leading to transepidermal water loss and invasion of bacteria, viruses, and allergens (69).

Two hypotheses concerning the initial event in the pathogenesis of AE have been proposed (70): One states that epithelial-barrier dysfunction is the consequence of the local inflammation. The other considers intrinsic defects in the epithelial cells as the cause of barrier dysfunction, and the local inflammation induced by invading allergens, bacteria, and viruses as epiphenomenon.

Decreased expression of antimicrobial peptides such as defensins and cathelicidins (LL-37) favors the colonization of lesional and unaffected skin by different microorganisms, including bacterial and fungal species (71, 72). Particularly, infections with *Staphylococcus aureus* (*S. aureus*) were found to trigger AE: *S. aureus* stimulates the production of GM-CSF and CCL5 in keratinocytes (73), and releases enterotoxins, which

act as superantigens, induce the pruritic cytokine IL-31 in T cells (74, 75), and neutralize the suppressive function of Treg cells (76).

Beside microbial infections, also exposure to allergens triggers the cutaneous inflammation. While children are mainly sensitized against food allergens such as milk, egg, wheat, or soy, adults frequently show specific IgE antibodies against house dust mites or the skin colonizing yeast *Malassezia* (77, 78). *Malassezia sympodialis* (*M. sympodialis*) is found on the skin of healthy individuals as well as on unaffected skin and inflammed lesional skin of AE-patients. However, 30-80% of AE-patients are sensitized against *M. sympodialis* and IgE specific for *Malassezia* was found to be restricted to atopic and non-atopic eczema. Sensitization to this particular yeast does not occur in any other allergic diseases (78). To date 3 allergens from *M. furfur* and 10 from *M. sympodialis* are known. While some allergens like Mala s 1 are species-specific (79), others are phylogenetically highly conserved. Mala s 6 (80), Mala s 11 (81), and Mala s 13 (82) show sequence similarity and cross-reactivity with the corresponding human proteins (see chapters 5.6.3). Autoreactivity to human self-antigens due to molecular mimicry with environmental allergens might be of pivotal importance for the exacerbation of chronic AE (see chapter 5.6.4) (83).

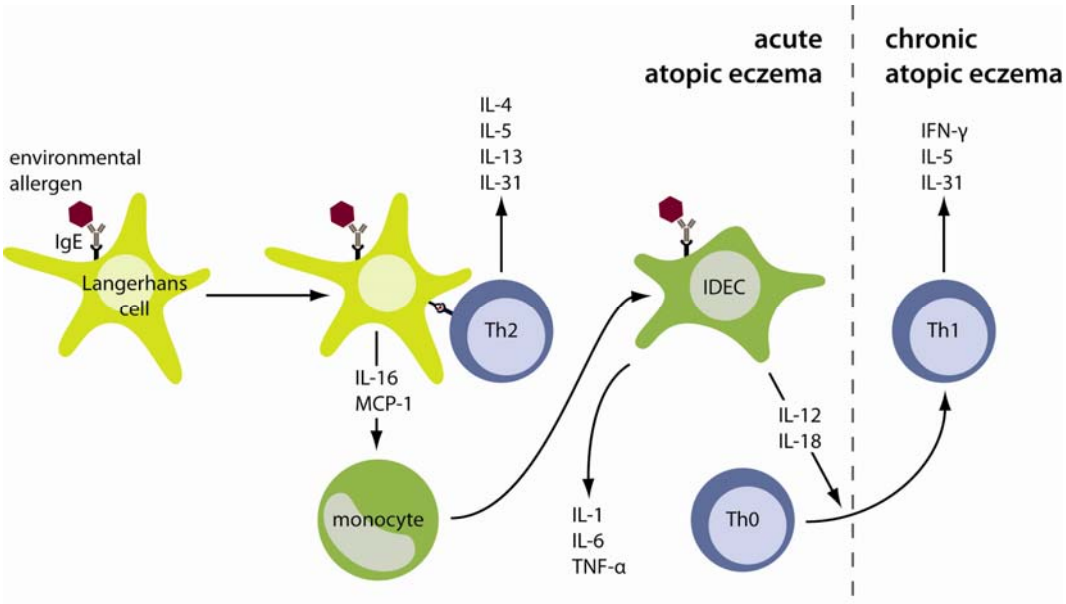

Figure 4. Acute and chronic phase of AE. In the acute phase of AE, binding of allergens activates Langerhans cells that present allergen-derived peptides to T cells and induce a Th2 profile. The secretion of IL-16 and MCP-1 attracts monocytes. After migration into the skin the recruited monocytes differentiate into IDECs. By the secretion of IL-12 and IL-18 IDECs favor the differentiation of Th1 cells, which constitute the major T cell subset during chronic atopic eczema. (Adapted from Bieber T et al. (70))

AE has a high familiar occurrence; studies of twins with AE have demonstrated concordance rates of 0.72 in monozygotic and 0.23 in dizygotic twin pairs (84). However, various genetic and epidemiological studies demonstrated that not a single gene can be implicated as the gene responsible for the development of AE, but various candidate genes and polymorphisms of immune regulatory genes, atopy related genes, and skin-specific genes play a role in the pathogenesis of AE. For instance mutations in the genes for filaggrin (85, 86) and stratum corneum chymotryptic enzyme (87) lead to impaired dermal differentiation, integrity and function of stratum corneum. Genetically based modifications of toll-like receptor (TLR) 2 (88), eotaxin (89), C3 (90), and RANTES (91) (92) or of cytokines like IL-4 (93), IL-18 (94), TGF-β (95), and GM-CSF (96) result in altered innate and adaptive immunity, respectively. Mapping of susceptibility loci identified by genome screens for AE, allergic asthma, and psoriasis demonstrated an overlap between susceptibility genes for AE and psoriasis but little overlap between AE and allergic asthma (97), indicating that specific genetic factors are involved in both diseases.

5.4.2 Allergic rhinitis

Allergic rhinitis is a frequent inflammatory disease of the upper airways resulting from the activation of mucosal mast cells beneath the nasal epithelium. Usually allergic rhinitis already begins in childhood, and often persists through adolescence and early adulthood, but tends to wane in older adults. The prevalence of allergic rhinitis was found to be in the range of 25% in European children aged 5-13 years (98). Thus, allergic rhinitis represents the most frequent allergic disease.

Two forms of allergic rhinitis are distinguished by time of allergen exposure: Seasonal allergic rhinitis is mainly triggered by pollen from trees, grass, and weed, while house dust mites, animal dander, cockroaches, and moulds are the cause of perennial allergic rhinitis. The immediate phase response occurs within minutes after allergen-exposure and induces repetitive sneezing, itching of the eyes, nose, and throat, and watery rhinorrhea. After 4-8 hours the late phase response begins, which is characterized by congestion, fatigue, and malaise. Although allergic rhinitis is not life-threatening, it has a important impact on the quality of life, mainly by sleep disturbance, which in turn leads to impaired concentration and work performance (99). Several epidemiological and clinical studies report a close relationship between allergic rhinitis and allergic asthma. Frequently allergic rhinitis during childhood is followed by adult-onset allergic asthma (100).

5.4.3 Allergic asthma

Compared to AE or allergic rhinitis, allergic asthma is a more serious syndrome leading to frequent emergency department visits and 13 deaths per million in the United States in 2004 (101).

The acute phase of allergic asthma is a type I hypersensitivity reaction, initiated by an allergic response to a specific allergen, such as pollen, house dust mites, animal dander, or mould spores. The activation of antigen-specific Th2 cells and submucosal mast cells in the lower airways leads within minutes to severe narrowing of airway lumen by bronchial constriction and increased secretion of fluid and mucus, manifesting by wheezing, coughing, dyspnea, and chest tightness. The acute phase is followed by a chronic phase of allergic asthma, which is characterized by a type IV hypersensitivity reaction, the infiltration of Th2 and Th1 cells into the lung, and airway remodelling due to hyperplasia and hypertrophy of smooth muscle cells, apoptosis of airway epithelial cells, goblet-cell metaplasia, and fibrosis.

While the acute phase of allergic asthma is initially driven by allergen-exposure, the chronic inflammation is also perpetuated by exposure to cigarette smoke, diesel exhaust particles, exercise, and viral or bacterial infections in the absence of specific allergens (102).

5.4.4 Allergic bronchopulmonary aspergillosis

Allergic bronchopulmonary aspergillosis (ABPA) was first described in the 1950s as a hypersensitivity reaction to the moulds *Aspergillus fumigatus* (*A. fumigatus*), *Aspergillus niger*, and *Aspergillus flavus* that colonize the bronchi (103). In contrast to invasive aspergillosis, which is mainly observed in immunocompromised individuals, ABPA occurs in immunocompetent individuals; 7-14% of corticosteroid-dependent asthmatics or cystic fibrosis patients develop ABPA (104, 105). *Aspergillus* spores are ubiquitous in soil and are commonly found in the sputum of healthy individuals. Upon inhalation of the spores, they germinate in the patients' airways and elicit Th2-medited asthmatic reactions. The release of mycotoxins and proteolytic enzymes by the fungus leads to mucus secretion and bronchiectasis, characterized by irreversible destruction and widening of the airways (106).

Interestingly, certain *A. fumigatus* allergens such as Asp f 4 and Asp f 6 are specific markers for ABPA and thus, recombinant Asp f 4 and Asp f 6 allow the diagnostic discrimination between ABPA and simple *A. fumigatus* allergy (107).

5.4.5 Allergic contact dermatitis

Allergic contact dermatitis is a cell mediated type IV hypersensitivity reaction, affecting around 20% of the population with nearly the same prevalence among children and adolescents (108). Upon exposure to a contact allergen, antigen-specific Th1 cells activate macrophages to secrete chemokines, cytokines like IL-1 or TNF-α, and cytotoxins like nitric oxide that lead to tissue damage and allergic symptoms such as local redness, edema, warmth, and pruritus. Many contact allergens are known, including latex, poison ivy, poison oak, nickel, and various substances present in cosmetic products and hair dyes as the most common ones (109). Although contact eczema may persist life-long, the eczema usually heals rapidly when exposure to antigen is avoided.

5.5 Epidemiology of atopic diseases

5.5.1 Prevalence and clinical significance

During the last century the prevalence of allergic diseases has tremendously increased worldwide and thus allergy is frequently referred to as epidemic of the 21st century. For instance, between 1979 and 1990 the hospitalizations of asthmatic children has increased 20-fold in the United States (102). While 1964 around 19% of Australian children were found to suffer from asthma or wheezing at some time during their first 7 years of life, in 1990 such symptoms were reported for 46% of children (110).

In general, allergic asthma, AE, and allergic rhinitis are more common in Western societies compared to developing countries. Phase II of the International Study of Asthma and Allergies in Childhood (ISAAC) reports a prevalence of asthma between 2% and 3% among 6 to 7 year-old children in Indonesia or Ethiopia, whereas 28% of Australian and 23% of British children ever had asthma (111). In 2007 ISAAC Phase III showed that the asthma prevalence still rises in regions where the prevalence was low but decreases or remains stable in most regions where the prevalence was previously high (112). Several factors are discussed to account for the increasing prevalence of allergy, such as exposure to tobacco smoke, diesel exhaust particles, or air pollution, increased exposure to allergens, and changes in lifestyle accompanied with decreased levels of exposure to microbial substances or infections. The relationship between hygiene, exposure to microbial sources, and allergy led to postulate the hygiene hypothesis (see chapter 5.5.2).

Although allergic diseases, except insect venom allergy, are rarely life-threatening they markedly reduce the quality of patients' life and cause much distress and financial costs. Considering the direct costs for life-long medication as well as the indirect costs by lost

time from work and reduction of productivity, allergic diseases account for more than € 35 billion health care burdens only in Europe per year (113).

5.5.2 The hygiene hypothesis

Allergic disorders clearly have a heritable component; however, the rapidity of the epidemiological changes indicates that primarily environmental factors are responsible for the recent rise in allergy prevalence. The hygiene hypothesis states that a reduction of infectious stressors during early childhood leads to increased longevity on the one hand, and to increased prevalence of allergy on the other hand (114). Infectious and non-invasive microbial exposure is supposed to guide the development of the immune system during the first years of life. Microbial compounds activate the innate immune system via TLRs. TLR-signaling pathways are believed to lead to the expansion of Th1 cells and at the same time to the suppression of the generation of Th2 cells, IgE, and Th2-dependent diseases (115). Supporting this hypothesis various epidemiological studies found an inverse correlation between atopy and the number of older siblings, early day-care attendance, the use of antibiotics, residence on a farm, and the consumption of unpasteurized cow's milk early in life (116, 117).

In addition helminth infections are found to prevent the development of allergy (118) and anti-helminth therapy is reported to lead to a significant increase in allergic skin sensitization among children from Venezuela and Gabun (119, 120). Helminths are supposed to activate a systemic anti-inflammatory network by the upregulation of IL-10 and TGF-β and the induction of Tregs (118). Moreover, parasitic nematodes secrete ES-62, a TLR-4 agonist, which inhibits the FcεR-induced release of allergy mediators from mast cells (121).

Although the hygiene hypothesis is considered as a valid model to explain many of the observed differences in asthma prevalence among populations, some recent studies point out its limitations: Changes in the asthma prevalence were described while the way of life and the housing conditions were kept constant (122, 123). Not all early childhood infections inhibit the tendency to develop allergic diseases and some respiratory virus infections are even positively associated with the development of asthma (124, 125). Moreover, not all helminth parasites are found to have protective effects on the outcome of allergic diseases and studies on the interactions between helminths, allergy, and immune responses show variable results depending on the species of helminths, the age when the infection was acquired, and the intensity of infection (118).

5.6 Allergens

Allergens are substances able to induce a primary sensitization, to bind IgE antibodies, and to elicit an allergic reaction. Most allergens are proteins, but also haptens such as the penicilloyl group, or certain glycosidic side chains from plant proteins, insect venom, or seafood were found to bind IgE.

Today more than 700 allergens have been identified and their sequences determined, including pollen, plant, and food allergens as well as indoor allergens from house dust mites, animal dander, and moulds. The list of described allergens is still continuously growing and updated information can be retrieved from databases such as the Official List of Allergens issued by the International Union of Immunological Societies Allergen Nomenclature Sub-committee (http://www.allergen.org), the Allergome database (http://www.allergome.org), the Food Allergy Research and Resource Program Allergen Database (http://www.allergenonline.com), or the InformAll database (http://foodallergens.ifr.ac.uk/). All allergens are named according to a standardized nomenclature, starting with 3 letters indicating the genus, followed by a single letter for the species, and a number for chronological order of allergen identification.

5.6.1 What makes an antigen an allergen?

To date it is not exactly known, why certain foreign proteins elicit allergic reactions, whereas other ones, which encounter the immune system via the same route of exposure, are tolerated. The allergenicity of a certain protein depends on its solubility, size, compactness of the overall fold, stability in the tissue, and the concentration in the corresponding allergen extract. Thus, they are subdivided into minor and major allergens. By definition a major allergen binds IgE in sera from more than 50% of the patients sensitized to the corresponding extract (126). Moreover, additional substances present at the time of allergen exposure either from the allergen extract itself such as lipids in pollen or from another source such as air pollution in the form of e.g. diesel dust or nanoparticles, or bacterial and viral infections can enhance the primary sensitization and secondary immune response to allergens.

All allergens described are heterogeneous in regard to their size and function. Structural and functional studies have failed to identify unifying properties or structural motifs characteristic for all allergens. Thus, for a long time it was considered that any antigen, which comes into contact with the immune system in sufficient amounts and in the appropriate context, might elicit an allergic response.

However, most allergens can be grouped into a small number of protein families and possess a limited range of biochemical functions such as hydrolysis of proteins, binding of metal ions and lipids, transport, storage, or they are associated with the cytoskeleton

(127, 128). The connection between biochemical function and allergenicity is best understood for Der p 1, the major allergen from the house dust mite. Der p 1 is a cystein protease that cleaves the tight junction protein occludin, leading to increased epidermal permeability and facilitated entry of Der p 1 into the tissue (129).

Today the prediction of allergenicity of a certain protein by bioinformatics is only possible to a very limited extend (130) but is of great interest when new proteins are introduced into our environment, for instance in genetically modified foods or as biological in medicine. Therefore the *in silico* prediction of allergenicity constitutes a challenging topic for future research.

5.6.2 Recombinant allergens

In 1988 Der p 1, the first recombinant allergen, was cloned (131) and since then numerous others followed. Today information about the allergen sequence together with cDNA from the allergen source allows the rapid cloning by PCR, recombinant production, and purification of allergenic molecules, which are crucial for elucidating the immunological mechanisms of allergy, but also for diagnostic and therapeutic use.

Since cloning of recombinant allergens necessarily depends on the availability of information on the allergen sequence, the identification of allergens from a given allergen source is of outmost importance. Screening of cDNA libraries with serum IgE from allergic patients allows the selective enrichment of clones expressing IgE-binding molecules and the determination of their primary structure. Frequently cDNA libraries displayed on phage surface are used to isolate new allergens such as allergens from *Aspergillus* (132), *Malassezia* (133), *Cladosporium* (134), peanut (135), wheat (136), and maize (136). Phage surface display technology exploits the linkage between phenotype, expressed as gene product displayed on the phage coat, and genotype, defined by the genetic information integrated into the phage genome. Moreover, phage display technology is adaptable to robotic based high-throughput screening technology, ensuring rapid and cost-efficient identification of a large number of IgE-binding antigens and fast determination on their sequences (137, 138).

The recombinant expression of allergens is possible in different host cells; the most appropriate one, however, has to be established for each protein considering its subsequent application. *Escherichia coli* (*E. coli*) is a suitable host for high-level expression of recombinant proteins, but lacks the capacity of post-transcriptional modifications, which sometimes are necessary for IgE-binding (139). Moreover, bacterial contaminants in purified protein solutions may interfere with cellular assays *in vitro* or lead to toxicity *in vivo* (140). Eukaryotic expression systems such as yeast cells (141), insect cells after baculovirus infection (142), or plant cells after tobacco mosaic virus

infection (143) might overcome these limitations and have been used for the production of glycosylated, pure recombinant allergens (144).

Several powerful techniques for purification of recombinant proteins are available, but the most frequently one used at laboratory scale is affinity chromatography, particularly immobilized metal affinity chromatography (IMAC), which allows efficient purification of recombinant His_6-tagged fusion proteins in a single step. Ion-exchange and size exclusion chromatography are suitable methods for further removal of contaminants deriving from host cells.

Both diagnosis and immunotherapy of allergic diseases are primarily based on aqueous allergen extracts. They consist of complex mixtures of proteins, while only a few of them are allergenic. Allergen extracts are difficult to standardize and large differences in allergen content between different batches of extracts and between extracts from different manufacturers were found (145). In contrast, recombinant proteins equal their natural counterparts in allergen extracts but are producible in consistent pharmaceutical quality under GMP conditions, avoiding problems associated with extract standardization (146, 147).

The diagnosis of allergic diseases is performed either by skin prick test *in vivo* or the detection of specific serum IgE antibodies *in vitro*. For both applications recombinant allergens were found superior to allergen extracts. A study including 156 patients with atopic eczema found IgE antibodies recognizing a *Malassezia furfur* extract or 6 recombinant *Malassezia furfur* allergens (Mal f 1, Mal f 5-9) in 47% or 55% of the patients, respectively (148). Future allergy diagnosis might be based on microarray technology, which allows simultaneous screening of small amounts of serum for IgE antibodies binding to panels of recombinant allergens (149, 150).

A detailed diagnosis allows determination of a component-resolved sensitization pattern, which allows patient-tailored allergen-specific immunotherapy (SIT), the only curative treatment for allergic diseases. SIT is used since almost 100 years and based on the repeated administration of the sensitizing allergen. Since different patients exhibit different patterns of allergen recognition, a patient-tailored immunotherapy with (combined) single recombinant allergens selected according to the patient's reactivity profile might improve therapeutic success compared to the use of a crude allergen extract. In addition immunotherapy with recombinant allergens allows the use of optimal concentrations of the relevant allergens while excluding allergens not recognized by the patient, and thus avoiding *de novo* sensitization to proteins supplied in suboptimal concentrations. Successful immunotherapy depends on the ability of the used vaccine to modulate the specific T cell response without binding IgE on the surface of effector cells (151), which bears the danger of inducing anaphylactic side-effects. Genetically

engineered recombinant proteins, so-called hypoallergens, meet these demands by reducing IgE-binding and adverse effects, while maintaining T cell reactivity and the therapeutic potential (152, 153).

5.6.3 IgE cross-reactivity of allergens

In clinical practice monosensitization is rarely observed because the majority of allergic patients reacts to various allergens. This is either due to polysensitization or to cross-reactivity among homologous molecules present in different allergenic sources. Cross-reactivity results from molecular mimicry mediated by sharing of common B or T cell epitopes by two or more antigens.

T cell epitopes are linear peptides consisting of 13-25 amino acid residues (154) whereas B cell epitopes are mostly conformational. They cover a surface area of 650-900 $Å^2$, involving 15-22 amino acid residues of which 5-6 are energetically important for antibody binding (155, 156). Hence, the structure of a protein is crucial for IgE-binding. Similar protein folds can be found among proteins with 25% sequence identity, while cross-reactivity is rare below 50% sequence identity (157).

Groups of phylogenetically conserved proteins from distant species showing IgE binding capacity are referred to as pan-allergens. The first identified representatives were the major birch pollen allergens Bet v 1 and Bet v 2. Bet v 1 homologues have been detected in other pollen (158), as well as in foods such as apple (159), pear, celery (159), carrot (160), peanut (161), hazel nuts (162), or cherry (163). Bet v 2 is a member of the protein family of profilins, which are highly conserved and responsible for cross-reactivity among botanically unrelated species of fruits, vegetables, and pollen (164, 165). Interestingly, Bet v 2 also shares structural similarity and cross-reacts with human profilin (166) (see chapter 5.6.3). Other families of pan-allergens are enolases (167), heat shock proteins (168), ribosomal (169) and peroxisomal proteins (170), manganese superoxide dismutases (MnSOD) (171), cyclophilins (172), and thioredoxins (173), which are responsible for cross-reactivity between phylogenetically different species, including pollen, foods, allergenic moulds, yeasts and even human self-antigens.

During the past few years the three-dimensional structures of an increasing number of allergens have been determined by X-ray crystallography or NMR spectroscopy. Although crystal structures have failed to elucidate the reasons why some proteins are allergenic and others are not, they have contributed substantially to our understanding of molecular interactions and thus cross-reactivity at the molecular level. It is possible to reliably predict cross-reactive B cell epitopes by homology modeling based on the comparison of 3D structures. Only amino acid residues, which are conserved in both primary sequences and at least partially exposed to solvent can account for cross-

reactive B cell epitopes. Superimposition of the crystal structures of the fungal allergens Asp f 6, Mala s 6, and Mala s 13 with the structures of human MnSOD, cyclophilin, and thioredoxin, respectively, allowed the identification of patches of conserved amino acid residues, which most probably constitute cross-reactive B cell epitopes (Figure 5) (80, 82, 171).

Site-directed mutagenesis can corroborate these results and elucidate the actual contribution of a single amino acid residue to IgE-binding and cross-reactivity (174). Moreover, these findings allow the rational design and production of a molecule with reduced IgE-binding capacity, useful as candidate vaccine that might induce desensitization to a whole group of pan-allergens.

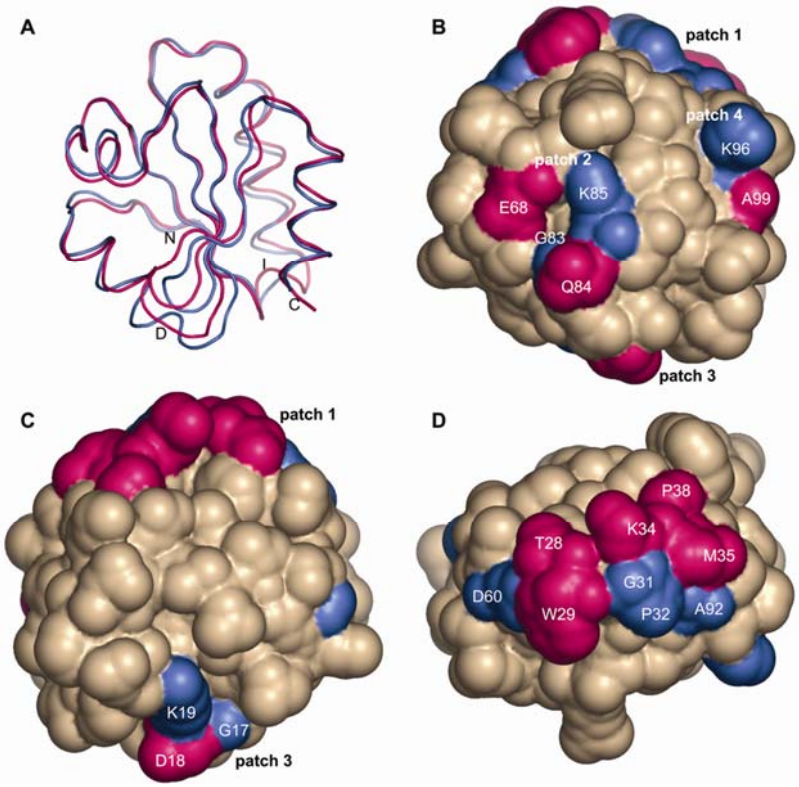

Figure 5. Prediction of cross-reactive B cell epitopes by homology modeling. A. Superposition of Mala s 13 (pink) on human thioredoxin (blue) reveals large structural similarities between the two homologous proteins. In Mala s 13 the first α-helix of is shortened due to a deletion of two amino acids (D), while an insertion of two additional residues leads to an additional small α-helix (I). The N- and C-termini are designated N and C, respectively. **B-D.** Predicted cross-reactive B cell epitopes are illustrated on the solvent-accessible surface of Mala s 13. Amino acid residues that are identical in Mala s 13 and human thioredoxin and at least 50% or between 30% and 50% solvent-exposed in both proteins are shown in pink or blue, respectively. (From Limacher A et al. (82))

5.6.4 IgE-reactive self-antigens

Already in 1941 it was demonstrated that human skin dander is able to trigger immediate type skin reactions in patients with severe AE (175). However, the observation that IgE-mediated autoreactivity might play a role in the pathogenesis of AE was not further followed, until modern molecular biology led to the identification of IgE-binding self-antigens. This allowed the explanation of the observed phenomenon on the basis of molecular mimicry and cross-reactivity.

The first described self-antigen was profilin (164), which was identified by data bank searches and found to cross-react with the major birch pollen allergen Bet v 2. To date several IgE-binding self-antigens associated with AE are described and most of them share high homology to conserved environmental allergens, such as human MnSOD (132), ribosomal protein P2 (169), cyclophilin (172), and thioredoxin (173). Moreover, four self-antigens, namely SART-1 (Hom s 1) (176), α-NAC (Hom s 2) (177), BCL7B (Hom s 3) (178), and cytokeratin type II (Hom s 5) (178) that do not share homology to known environmental allergens have been identified.

All these proteins were recombinantly produced and shown to bind IgE of sera of patients suffering from AE, to induce the proliferation of T cells, and to elicit immediate type I hypersensitivity reactions in skin test challenges in patients sensitized to the corresponding environmental allergen (83). In addition Hom s 2 is able to induce the release of IFN-γ in cultured peripheral blood mononuclear cells (PBMC) of atopic individuals, leading to disintegration of respiratory epithelial cell layers and apoptosis of skin keratinocytes (179).

The observed cross-reactivity between human self-antigens and environmental allergens raises the question of primary sensitization. Primary sensitization against human self-antigens is rather unlikely since most of the individuals examined so far are not sensitized exclusively to the self-antigen but also react to homologous environmental allergens (171). Thereby the IgE as well as the cellular immune response against the environmental allergen are usually stronger compared to the reaction against the endogenous counterpart (171). These observations support the assumption that autoreactivity is initiated by sensitization against an environmental allergen, leading to inflammation and tissue damage. Thereby intracellular proteins are released as a consequence of inflammatory processes and become accessible to the immune system. These proteins are supposed to cross-react with IgE-antibodies primarily raised against the homologous environmental allergens. In addition, cellular stress during the inflammatory response induces the upregulation of stress-inducible proteins such as MnSOD (81). In a Th2-prone environment self-antigens may induce the *de novo* production of IgE autoantibodies and cross-link IgE bound on mast cells or basophils

inducing mediator release. Autoreactive T effector cells may elicit apoptosis of keratinocytes via the secretion of IFN-γ and FasL and thus contribute to the perpetuation of the symptoms. The hypothesis that IgE-mediated autoreactivity plays a relevant role in the pathogenesis of chronic atopic diseases like AE is in accordance with clinical observations showing that exacerbation of the disease can also occur in the absence of exposure to environmental allergens (180).

6 Aim of the thesis

It has been known for more than 60 years that human skin dander can trigger immediate type skin reactions in AE patients. Moreover, in clinical practice exacerbations of the disease are frequently observed in the absence of environmental allergens. These observations might be due to cross-reactivity of human proteins showing structural similarity with phylogenetically conserved allergenic proteins. In fact, human homologues of environmental allergens are able to elicit type I skin reaction in patients sensitized to the corresponding environmental allergen, supporting a pathogenic role for IgE-mediated autoreactivity in AE. Several IgE-binding self-antigens such as MnSOD, cyclophilin, and thioredoxin are known and already characterized in deep molecular detail. However, the extent of the repertoire of IgE-binding self-antigens associated with, AE and their role in the pathogenesis are still largely unknown.

Therefore, the aim of this thesis was to study IgE-mediated autoreactivity in AE by

1. Identification of new self-antigens associated with AE by high-throughput screening of a human phage-surface display library with serum IgE from AE patients;
2. Cloning, recombinant production, and purification of selected human self-antigens;
3. Characterization of their immunological properties with respect to antibody-binding, induction of mediator release from basophils, and activation of T cells from sensitized AE patients;
4. Analysis of the expression of self-antigens in the skin of AE patients and healthy individuals.

7 Results

7.1 Exploring the repertoire of IgE-binding self-antigens associated with atopic eczema

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Abstract

Background: Atopic eczema is the most common chronic inflammatory skin disease. Recent data demonstrate the presence of autoreactive serum IgE antibodies correlating with the severity of the disease.

Objective: Although several IgE-binding self-antigens have been reported, the whole repertoire of IgE-binding self-antigens is unknown. We aimed to estimate the repertoire size of autoreactive proteins related to atopic eczema, clone, produce, and characterize humoral and T cell responses against novel self-antigens.

Methods: Phage surface displayed human cDNA libraries were enriched for clones binding to serum IgE from atopic eczema patients and screened by high-throughput technology. Selected clones were used to produce the encoded proteins for further testing their IgE-binding ability in Western blots and ELISA, to induce proliferative responses in PBMC, and mediator release from basophils of sensitized individuals.

Results: 140 sequences encoding potential IgE-binding self-antigens associated with atopic eczema were identified. 16 sequences encoded already described self-antigens. 3 new sequences showed homology with environmental allergens, 86 encoded known human proteins, 7 predicted proteins, and 28 showed sequence identity with genomic contigs. Immunoblotting and ELISA experiments showed the presence of IgE-antibodies in sera from atopic eczema patients to five selected recombinant self-antigens. They were able to induce PBMC proliferation and mediator release from basophils of atopic eczema patients who have self-antigen-specific IgE antibodies.

Conclusion: The data demonstrate a broad spectrum of at least 140 IgE-binding self-antigens associated with atopic eczema. By binding IgE-antibodies or activating specific T cells they might promote and/or perpetuate existing skin inflammations.

Introduction

The description of “autoreactivity” goes back to the early last century when the sensitivity of individuals to human skin dander was demonstrated (175, 181). The observation that autoreactivity might play a role in the pathogenesis of atopic eczema (AE) was not further followed, until progress in molecular biology allowed isolation of cDNAs encoding IgE-binding proteins (182, 183) and production of recombinant self-antigens (184). Interestingly most of these autoantigens have been isolated using sera of patients suffering from AE (178, 185). AE is a chronic relapsing inflammatory skin disease characterized by severely itchy, red, dry, and crusted skin and has a great impact on life quality of affected individuals. The pathogenesis of AE is likely to result from a combination of a defective skin barrier and inappropriate immune responses with contribution of genetic and environmental factors (186, 187). Environmental triggers for AE can be different life style factors, stress, allergens, and microorganisms (186). In addition, there is increasing evidence that IgE-mediated reactions to self-antigens could play an essential role in the pathogenesis of AE (188, 83). Recombinant human self-antigens with high homology to environmental allergens like manganese-dependent superoxide dismutase (MnSOD) (132, 189), ribosomal P_2 protein (169), cyclophilin (172, 80), and thioredoxin (173, 82) have been shown to bind IgE from sera of AE patients , elicit proliferation of peripheral blood mononuclear cells *ex vivo*, and induce immediate type I hypersensitivity skin reactions exclusively in patients sensitized to the corresponding homologous environmental allergen. Moreover, human MnSOD, which alone elicits an eczematous reaction in atopy patch tests, is up-regulated in lesional skin of AE patients (81). These data provide strong evidence for a causal implication of autoreactivity in the pathogenesis of AE.

Other human self-antigens described as Hom s 1 (176), Hom s 2 (179), Hom s 3 (188), and Hom s 5 (188) lack sequence homology to known environmental allergens. In contrast, Hom s 4 shows homology to a subfamily of calcium-binding allergens from plants and fish (190). However, all these self-antigens are able to cross-link specific IgE on effector cells as demonstrated by their ability to elicit immediate type I skin reactions. Recently it has been shown that Hom s 2-stimulated peripheral blood mononuclear cells (PBMC) of atopic and non-atopic individuals secrete IFN-γ, which mediates damage of epithelial cells and apoptosis of keratinocytes (179). Strong type I skin reactions, induction of keratinocyte apoptosis, and the observation that the level of specific autoreactive IgE-antibodies correlate with the severity of the disease (81) further suggest the involvement of autoreactivity in the pathogenesis of AE.

Although several self-antigens have been described (83) and some characterized in deep molecular and structural details (189) (80) (82), the whole spectrum of IgE-binding self-

antigens associated with AE is still largely unknown. Therefore, we probed the repertoire of IgE-binding self-antigens by screening a human cDNA library displayed on phage surface with serum IgE of AE patients. High-throughput screening of cDNA libraries displayed on page surface by affinity selection and high-density arrays is a rapid method for the identification of IgE-binding clones (191) and yielded during this work a long list of 140 discrete sequences potentially encoding IgE-binding proteins. Seven sequences, including the known self-antigens cyclophilin B and thioredoxin as positive controls, were chosen to test the specificity of the enrichment procedure through cloning, production, and characterization of the recombinant self-antigens.

Methods

Construction and screening of human phage surface displayed cDNA libraries

A human cDNA library was constructed from human lung mRNA in phagemid pJuFo3 and displayed on the surface of filamentous phage M13 as described (191) (137). Affinity selection of phages displaying human proteins was performed by four consecutive biopanning rounds against three different serum IgE pools from AE patients immobilized to microtiter plate wells (Maxisorp^{TM}; Nunc, Roskilde, Denmark) through passively absorbed mouse anti-human IgE mAb TN142 (137). After the fourth round, eluted phagemids from the three selections were independently plated on 20 x 20 cm square plates at a density of 5-10'000 colonies/plate and grown overnight at 37°C. 2688 single colonies for each phagemid population were robot-picked and arrayed onto 7 medium-filled 384 well plates. After overnight growth, cDNA inserts of all picked clones were amplified by high-throughput PCR using a PTC-225 thermal cycler (Tetrad, MJ Research Inc., Watertown, MA) and gridded in duplicates onto filter membranes to produce hybridization filters. 24 Filter hybridizations using DIG-labeled PCR probes derived either from known self-antigens or from the sequences of randomly selected clones were performed as described (192). Positive clones were scored using the image analysis package VisualGrid (GPC, Munich, Germany). Matrices of hybridization patterns were compared using the program Hybcompare (191). Inserts and PCR products were sequenced using dye-terminators on an automated sequencer (ABI-Perkin Elmer, Foster City, CA). Homology searches were performed with BLAST and the Genetic Computer Group program FASTA (193).

Cloning, production, and purification of recombinant human proteins

The coding sequences of the selected human proteins were amplified by PCR from a commercial human lymphoma cDNA library (U937, Stratagene, La Jolla, CA) with sequence-specific primers (Table 2), subcloned into the high-level expression vector pET17b (Novagen, San Diego, USA) and transformed into *E. coli* BL21 (DE3) star pLysS strains (Invitrogen, Groningen, The Netherlands). After verification by DNA sequencing, clones containing correct inserts were used for the production of His_6-tagged recombinant proteins. Exponentially growing cultures were induced with 1 mM isopropyl-β-*D*-thiogalactoside (Fermentas, Burlington, Canada) at an OD_{600} of 0.6. After 5 hours further incubation at 37°C cells were harvested by centrifugation (4000 x g, 10 min, 4°C) and lysed under denaturing conditions. To desalt and reduce LPS contaminations the cleared *E. coli* lysate was applied to Sephadex G-25 columns (GE healthcare, Chalfont St. Giles, UK). Subsequently, recombinant proteins were purified via Ni^{2+}-chelate affinity

chromatography conditions using 5 ml HisTrap FF columns (GE healthcare) as described (194). To obtain soluble proteins, eluted fractions were dialyzed against ultra-pure water. Molecular size and purity of the His_6-tagged fusion proteins were determined by SDS-PAGE (NuPAGE; 12% Bis-Tris, Invitrogen).

Endotoxin concentrations were determined by limulus amoebocyte lysate assay (Cambrex, Walkersville, USA).

human protein	GenBank Acc. No.	primer		MW (kDa)
actin-α	NM_001613	5'-primer: 3'-primer:	5'-cgcggatccatgtgtgaagaagaggacagc-3' 5'-cccaagcttagaagcatttgcggtggac-3'	42
tubulin- α	NM_006009	5'-primer: 3'-primer:	5'-cgcggatccatgcgtgagtgcacttccatc-3' 5'-cccaagcttagtattcctctccttcttcc-3'	50
eIF6	NM_002212	5'-primer: 3'-primer:	5'-cgcggatccatggcggtccgagcttcg-3' 5'-cccaagcttaggtgaggctgtcaatgagg-3'	27
HLA-DR- α	K01171	5'-primer: 3'-primer:	5'-cgcggatccatcaaagaagaacatgtgatcatcc-3' 5'-cccaagcttacagaggccccctgc-3'	24
RP1	X94232	5'-primer: 3'-primer:	5'-agggaattccctgggccgacccaaacc-3' 5'-cccaagcttcagtactcttcctgctgcg-3'	39
cyclophilin B	NM_000942	5'-primer: 3'-primer:	5'-ggggatccatgaaggtgctccttgccgccgcc-3' 5'-cccaagcttctactccttggcgatggc-3'	22
thioredoxin	X77584	5'-primer: 3'-primer:	5'-cgggatccatggtgaagcagatcgagagc-3' 5'-cccaagcttagactaattcattaatggtggcttccagcttttcc-3'	12

Table 2. GenBank accession number (Acc. No.), PCR primer pairs used for cloning, and predicted molecular weight (MW) of selected human self-antigens. The recognition sites of BamHI (ggatcc), EcoRI (gaattc) and Hind III (aagctt) restriction enzymes used for cloning are underlined. Acc. No.: Accession Number; MW: molecular weight

Western blot analyses

Recombinant proteins were separated on NuPAGE 12% Bis-Tris polyacrylamid gels (Invitrogen) under reducing conditions and transferred to Hybond-P PVDF membranes (GE healthcare). Non-specific binding sites were blocked with 5% skimmed milk in PBS, 0.5% Tween-20 prior incubation of the membranes with sera, followed by primary mouse anti-human IgE mAb TN142 (195) and secondary PO-labeled sheep anti-mouse IgG mAb (GE healthcare). Bound IgE was visualized using ECL Plus Western Blotting Detection Reagents (GE healthcare).

IgG, IgG1, IgG3, IgG4, IgA, and specific IgE ELISA

Specific binding of serum IgG, IgG1, IgG3, IgG4, IgA, and IgE to recombinant human proteins was analyzed by direct solid phase ELISA. Polystyrene microtiter plates (Maxisorp) were coated with 5 µg/ml of recombinant protein and processed as described (196). Bound antibodies were detected with a primary antibody specific for the different isotypes: AP-conjugated goat anti-human IgG (Pierce, Rockford, USA); NL16 mouse anti-human IgG1 (Oxoid, Hampshire, United Kingdom); PO-conjugated mouse antihuman IgG3 (Zymed, Wien, Austria); RJ4 mouse anti-human IgG4 (Oxoid); PO-conjugated rabbit anti-human IgA (Dako, Glostrup, Denmark); TN142 mouse anti-human IgE (195). IgG1, IgG4, and IgE were visualized with a secondary goat AP-conjugated anti-mouse IgG(H+L) antibody (Pierce). After addition of the specific substrates (1.5 mg/ml di-Sodium 4-nitrophenyl phosphate (Sigma-Aldrich, St. Louis, USA) in Diethanolaminbuffer pH 9.8 (AP-conjugated antibodies) or 1.5 mg/ml o-phenylenediamine dihydrochloride (Sigma-Aldrich) in citrate buffer (PO-conjugated antibodies), absorbency was measured at 405 nm.

Activation of basophils

100 µl of heparinized venous blood was stimulated with recombinant self-antigens (1 µM), 0.9% NaCl or anti-IgE monoclonal antibody (1 µg/ml) at 37°C for 20 min. After inhibiting Ca^{2+}-dependent cell-signaling with 20 mM EDTA, the cells were stained with anti-CD63-FITC and anti-CD203c-PE or anti-IgG1-FITC and anti-IgG1-PE (all from Beckman Coulter, Fullerton, USA) as isotype control, and analyzed by flow cytometry (EPICS XL-MCL, Beckman Coulter). Basophils were identified by the expression of CD203c (197) and activation of the cells was analyzed by the up-regulation of CD63 (198). Flow-Count Fluorospheres (Beckman Coulter) were used to determine absolute cell counts.

Patients and control subjects

71 adult patients suffering from AE, 24 healthy individuals and as an additional control group 12 patients with psoriasis of both sexes were recruited and carefully examined regarding clinical history, clinical symptoms, medications, or other treatments. Atopic and non-atopic eczema were diagnosed according to the criteria of Hanifin and Rajka (199) and the EAACI recommendations (60). Severity of the eczema was determined by the SCORAD index (200). Total and allergen-specific serum IgE-levels were analyzed by the ImmunoCap system (Phadia, Uppsala, Sweden) and ELISA, respectively. Patients' characteristics including sex, age, diagnosis, SCORAD, and total serum IgE levels are reported on Table 3. Patients with total serum IgE levels > 100 kU/l and negative specific IgE and skin prick test to house dust mite, tree pollens, grass pollens, weed pollens, and

mould extract were assigned to the group of non-atopic eczema. The characteristics of this group of patients are reported on a separate Table (Table 4 in the Online Repository). The study protocol was approved by the ethics committee of the University of Zürich. A full explanation of the procedure was given to all participants, and their written consent was obtained before starting.

Statistical analysis

All statistical analyses were performed in GraphPad Prism 5. Patients' characteristics were compared using unpaired t-test, correlations between different antibody-isotypes determined by calculating Spearman's rank correlation coefficient.

Results

Identification of cDNA clones potentially encoding IgE-binding self-antigens

Screening of a human phage surface displayed cDNA library with serum IgE from three different pools of AE patients, yielded large populations of phage putatively expressing IgE-binding self-antigens. In the first screening round a set of filters were consecutively hybridized with dioxigenin-labelled probes derived from the known autoallergens MnSOD (189), P_2 ribosomal protein (169), and profilin (166). 2342 (29%) of the 8064 analyzed clones hybridized with the known sequences. Direct sequencing of the inserts from 50 randomly selected hybridization negative clones yielded 16 new sequences. Consecutive hybridization of the arrayed library with labeled probes of these 16 inserts allowed the identification of additional 3766 (46.7%) hybridization positive clones. Sequencing of further 50 hybridization negative clones yielded 22 new, so far undetected sequences, which were used in a third hybridization round resulting in the identification of additional 1728 (21.5%) hybridization positive clones. The remaining 228 hybridization negative clones (2.8%) were PCR amplified and directly 5'-sequenced. 72 clones (0.9%) gave no amplification products and among the remaining 156 clones (1.9%), 110 new sequences were found. Thus, after three rounds of hybridization including three already known sequences and 246 sequencing reactions, 99.1% of the 8064 clones could be assigned to 151 discrete sequences.

Characterization of the sequences

The 148 new sequences detected in addition to the known MnSOD, P_2 ribosomal protein, and profilin, were submitted to blast analysis to tentatively assign the putative self-antigens to known proteins. Ten sequences lacked to show relevant sequence identity to known proteins, probably due to the very short lengths of the ORFs encoded, and one clone resulted to be a contaminant (Table 5 in the Online Repository). The remaining 137 sequences represented sequences present in the human genome. Among these 27 sequences matched perfectly to genomic contigs derived from different chromosomes, one to the mitochondrion genome, three to chromosomal ORFs encoding putative proteins of 140, 194, and 416 amino acids, respectively, and four to predicted proteins. These sequences were not further investigated. The remaining 99 sequences, together with the known self-antigens MnSOD, P_2 ribosomal protein, and profilin, build a potential repertoire of IgE-binding self-antigens involving at last 102 distinct human proteins (Table 5 in the Online Repository).

A first group of 16 sequences coding for human proteins were already described as autoreactive self-antigens. Among these actin-α, tubulin-α, translation initiation factor 6

(eIF6), HLA-DR-α, and RP1 were demonstrated to be IgE-binding self-antigens and further investigated in the present study.

A second group includes calmodulin, myosin-1C, and transglutaminase. These three proteins, although not yet described as IgE-binding self-antigens, show homology to described environmental allergens from pollen (201), *Blattella germanica* (202), and *Dermatophagoides farina* (203). The largest group of 86 IgE affinity-enriched sequences coded for described human proteins without sequence homology to known allergens.

As expected from the screening of a cDNA library, not all sequences span complete open reading frames. In fact, only six of the 102 sequences coding for known human proteins represented full length clones (Table 5 in the Online Repository). However, the fact that all described IgE-binding self-antigens were present among the selected clones indicates that the majority of the detected sequences could indeed code for IgE-autoreactive self-antigens.

Validation of selected IgE-binding self-antigens

Cloning and production

Three out of the newly identified potential self-antigen sequences, namely actin-α, eIF6, and RP1 were detected only as single hybridizing spots among the 8064 clones analyzed. Two clones showed higher hit frequencies of 291 (HLA-DR-α) and 237 (tubulin-α). These five clones were selected for further investigations together with cyclophilin B (172) and thioredoxin (82), two well-known self-antigens, included as positive controls.

The sequences were amplified by PCR using gene specific primers (Table 2), subcloned as His_6-tagged fusion-proteins into the high-level expression vector pET-17b and recombinantly expressed in *E. coli* BL21 (DE3) star pLysS. After purification by Ni^{2+} affinity chromatography, size and purity of the recombinant proteins were tested by SDS-PAGE and Coomassie Blue staining. Production yields varied between 5 and 30 mg pure protein per liter culture, depending on the clone. The estimated molecular masses for the His_6-tagged proteins were in good agreement with the values calculated from the amino acid sequences and were virtually pure (Figure 6a). LPS contaminations of the recombinant protein preparations were found to be in the range of 0.1 to 0.2 EU/µg protein.

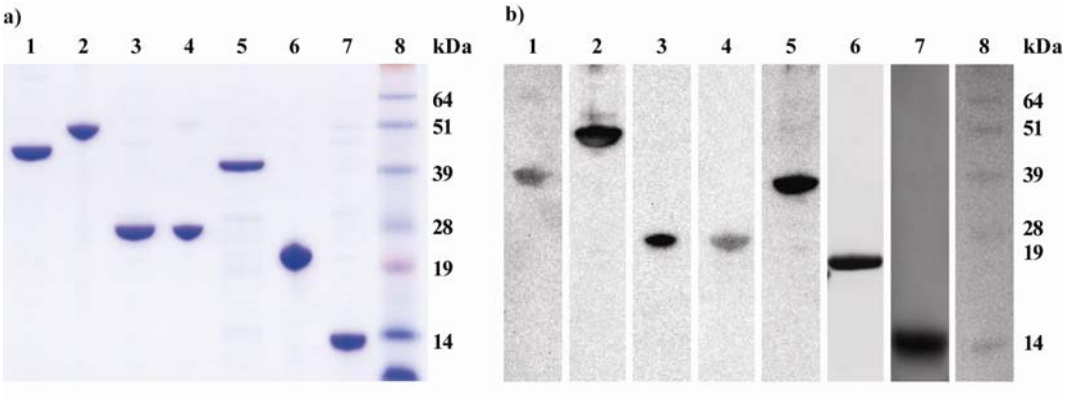

Figure 6. a) Recombinant proteins (2.5 µg) were separated by SDS-PAGE and stained with Coomassie Blue. **b)** Specific IgE-binding of the recombinant self-antigens analyzed by Western blotting using sera from different individuals suffering from AE.
Lane 1: actin-α; lane 2: tubulin-α; lane 3: eIF6; lane 4: HLA-DR-α; lane 5: RP1; lane 6: cyclophilin B; lane 7: thioredoxin; lane 8: molecular weight standard (kDa).

IgE-binding in Western blot and ELISA

The IgE-binding capacity of the selected self-antigens was first confirmed in Western blot using sera of AE-patients (Figure 6b).The prevalence of sensitization against the recombinant proteins was determined by ELISA (Figure 7). Sera of 71 AE patients and 24 healthy controls were analyzed for IgE-binding to the pure recombinant proteins. Signals were considered positive when the measured A_{405} value was more than 3-fold higher than the mean A_{405} value of the healthy controls for the respective self-antigen. Applying these cut-off values 51 out of 71 (71.8%) AE patients showed variable patterns of IgE autoreactivity to one or more of the tested self-antigens, while none of the healthy controls and of the psoriasis patients used as a control group for a non-IgE related chronic inflammatory skin disease showed specific IgE above background against any of these proteins (Table 3). These data indicate that IgE-mediated autoreactivity is confined to patients suffering from AE. In detail, 15.5%, 21.7%, 25.4%, 8.7%, and 29.0% of the AE-patients had specific IgE for actin-α, tubulin-α, eIF6, HLA-DR-α, and RP1, respectively (Figure 7). The prevalence of sensitization against the known self-antigen cyclophilin was 9.2%, whereas 23.4% of the analyzed patients had thioredoxin-specific serum IgE. These values are in good agreement with the prevalence reported in previous studies (80) (82) (204).

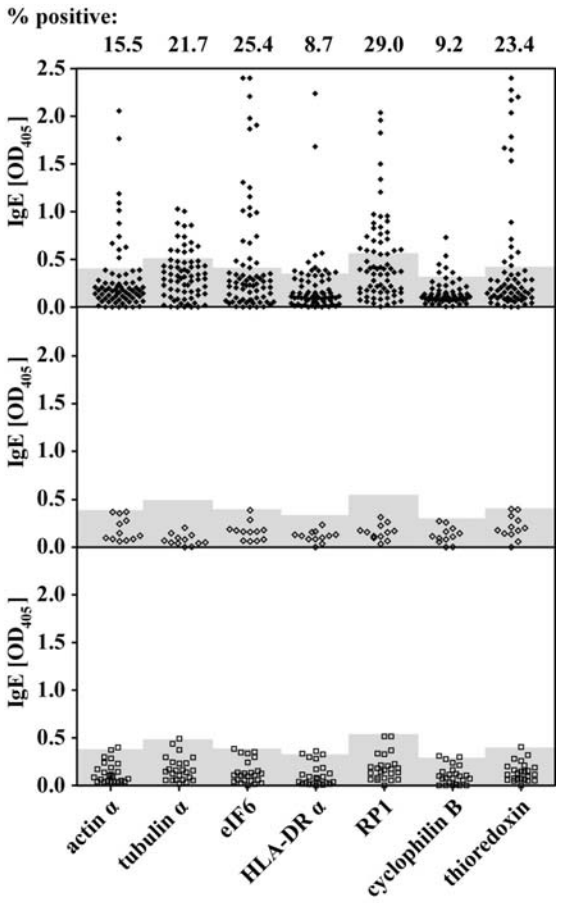

Figure 7. IgE specific for human self-antigens. Specific IgE against recombinant self-antigens determined by ELISA in sera from 71 AE-patients (◆, upper panel), 12 psoriasis patients (◇, middle panel) and 24 healthy controls (□, lower panel). The cut-off values for positive results are highlighted in grey and correspond to 3-times the mean of the healthy control group.

	AE patients		psoriasis patients	healthy controls
	with IgE to self-antigens	without IgE to self-antigens		
number	51	20	12	25
sex, female : male	30 : 21	12 : 8	6 : 6	9 : 15
mean age, y	33.35 ± 12.70	39.35 ± 13.5	45.17 ± 9.20	29.00 ± 3.71
total IgE, kU/ml	2598.05 ± 3749.52	876.95 ± 1547.93	87.14 ± 24.73	56.8 ± 38.52
SCORAD	47.43 ± 23.93	44.12 ± 22.19	0	0
atopic : non-atopic eczema	38 : 13	15 : 5	-	-

Table 3. Characteristics of AE patients with and without IgE specific to self-antigens, psoriasis patients, and healthy controls. AE patients with autoreactive IgE antibodies show no differences in mean age ($p \leq 0.7095$) and SCORAD index ($p \leq 0.7625$) but have significantly elevated levels of total IgE ($p \leq 0.0002$) compared to AE patients without autoreactive IgE antibodies.

Beside IgE, the antibody-isotypes IgA, IgG, IgG1, IgG3 (Figure 10 in the Online Repository), and IgG4 (Figure 8) specific for eIF6 were analyzed. All AE-patients and healthy individuals showed detectable levels of eIF6-specific IgG in serum. However, AE-patients had clearly elevated levels of specific IgG4 and IgA, while the levels of total IgG, specific IgG1, and specific IgG3 were comparable to healthy individuals. A significant positive correlation was found between the levels of specific IgE and IgG4 (Figure 8b) and between specific IgE and IgG (Figure 10b in the Online Repository).

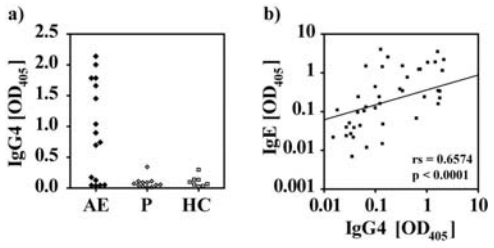

Figure 8. IgG4 specific for human eIF6. a) Specific IgG4 against recombinant eIF6 determined by ELISA in sera from 8 healthy controls (□, HC), 12 psoriasis patients (◇, P), and 16 AE-patients (◆, AE). **b)** Correlation between eIF6-specific IgG4 and IgE analyzed by ELISA in sera from 16 AE-patients and 8 healthy individuals. Spearman's rank correlation coefficient was calculated.

IgE reactivity to self-antigens in patients with non-atopic eczema

The mean age, ratio between males and females, and the SCORAD index were similar among the 51 patients with and the 20 patients without autoreactive IgE (Table 3). In contrast, total IgE values were significantly higher among the subset of patients sensitized to human self-antigens ($p>0.0002$). Interestingly, 13 of the 18 patients fulfilling the criteria for non-atopic eczema, defined as lack of sensitization to common environmental allergens and low levels of total serum IgE (60), showed IgE reactivity to self-antigens (Table 4 in the Online Repository). These patients might be sensitized to environmental allergens with sequence homology to self-antigens, which are not yet identified or might mount IgE responses as the result of a primary autosensitization.

Basophil activation and proliferative responses

The ability of the recombinant self-antigens to cross-link receptor-bound IgE on the surface of basophils was analyzed by the upregulation of CD63 on the surface of CD203c positive cells by flow cytometry. All recombinant self-antigens, except HLA-DR-α, were able to induce upregulation of CD63 on basophils from AE-patients sensitized to the corresponding self-antigen (Figure 9), suggesting that these proteins are indeed able to cross-link Fcε-receptors, which leads to mediator release and activation of the cells. In contrast, basophils from healthy individuals and from AE patients without antigen-specific serum IgE to self-antigens do not showed any upregulation of the CD63 surface marker upon stimulation with self-antigens (Figure 9). Moreover, PBMC of atopic patients sensitized to a given self-antigen showed specific proliferation upon stimulation with the antigen, whereas cells of healthy controls did not show self-antigen-specific responses (data not shown).

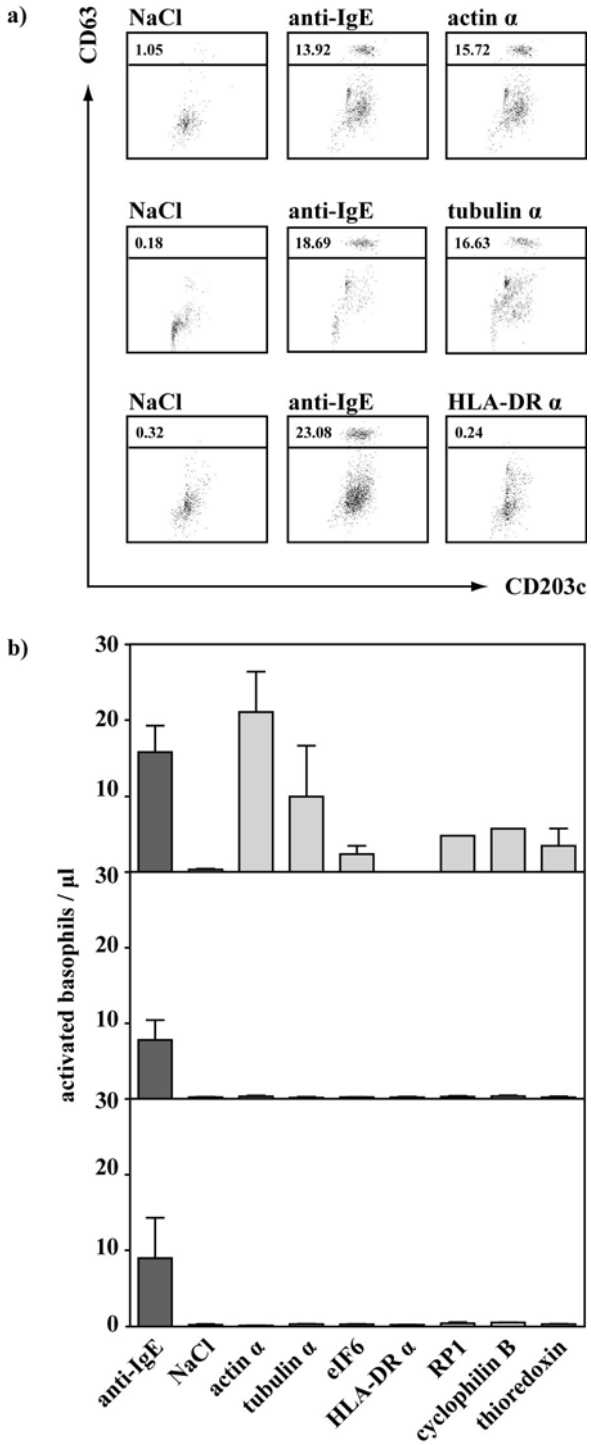

Figure 9. Activation of basophils. Numbers of activated basophils were analyzed by the expression of CD203c and CD63 after the stimulation with recombinant self-antigens, sodium chloride, or anti-IgE. **a)** Results of three representative AE-patients are shown. **b)** Self-antigen induced activation of basophils from blood of AE patients with self-antigen-specific IgE (upper panel), AE patients without self-antigen specific IgE (middle panel), and healthy controls (lover panel).

Discussion

Skin test reactivity to self-antigens is assumed to play a role in AE (175, 181) but only recently the application of modern cloning methods allowed the characterization of IgE-binding self-antigens at molecular level (188) (83). A first class of IgE-binding self-antigens, with profilin being the first member described (166), includes families of phylogenetically conserved proteins sharing sequence homology with environmental allergens. Recombinant self-antigens like MnSOD (132), ribosomal P_2 protein (169), cyclophilin (172), or thioredoxin (173) are able to bind serum IgE, induce T cell proliferation *ex vivo* in PBMC and strong skin test reactions exclusively in patients with serum IgE against the corresponding homologous environmental allergens (132, 169). Moreover, the demonstration that human MnSOD applied to healthy skin areas of AE patients is sufficient to elicit eczematous reactions (81) and that serum IgE autoantibodies target keratinocytes (204) strongly indicates a role of autoreactivity in the pathogenesis of AE. IgE autoreactivity to self-antigens with sequence homology to environmental allergens can be explained by shared B cell epitopes as shown by comparison of the solved crystal structures of environmental allergens and homologous human proteins (82, 80). However, Western blot analyses of protein extracts of human epidermis cell lines with serum IgE of AE patients revealed a large number of positive bands (204, 205). Screening of a λgt 11 expression cDNA library derived from human epithelial cells with serum IgE of AE patients allowed the identification of four cDNAs encoding self-antigens without significant sequence homology with known exogenous allergens (178). This raised the question about the size of the repertoire of IgE-binding self-antigens involved in AE, which should, according to the number of bands detected in Western blots, be much larger than those of the so far described molecules. In this work we aimed to estimate the repertoire size of IgE-binding self-antigens associated with the disease. High-throughput screening of a human cDNA library displayed on phage surface with serum IgE of AE patients revealed 140 diverse cDNAs potentially encoding autoreactive proteins. The presence of the majority of the so far described IgE-binding self-antigens among the selected clones points out that phage display is a suitable method for the fast and efficient isolation of self-antigens with affinity for serum IgE. To demonstrate the specificity of the selection, we cloned, expressed and investigated the IgE-binding capacity of five novel cDNAs coding for actin-α, tubulin-α, eIF6, HLA-DR-α, and RP1 that were detected either as single clones or at low frequency among the 8064 screened single clones (Table 5 in the Online Repository).

The prevalence of IgE sensitization among the AE-patients ranged from 8.7% to 29.0% (Figure 7), with the lowest frequency for HLA-DR-α and the highest for RP1, in good agreement with the frequencies reported for other IgE-binding self-antigens (83, 204).

Both AE-patients and healthy individuals had detectable levels of eIF6-specific serum IgG (Figure 10 in the Online Repository), supporting a previous study, which demonstrates IgG specific for the *Aspergillus fumigatus* allergen Asp f 3 in sera from healthy individuals as well as allergic patients (197). The levels of eIF6-specific IgG4 and IgA were elevated in sera from AE-patients compared to healthy controls and the levels IgG4 correlated directly with the levels of specific IgE (Figure 8).

Except HLA-DR-α, all tested recombinant proteins were able to cross-link receptor-bound IgE on the surface of basophils and upregulate the expression of CD63 and CD203c, which correlates with mediator release (Figure 9). As HLA-DR-α failed to activate basophils from sensitized individuals, it is possible that HLA-DR-α displays only a single epitope, leading to monovalent IgE-binding without cross-linking of Fcε-receptors. Elimination of autoreactive cells displaying more than one epitope could represent an efficient mechanism to avoid breakdown of immune tolerance to an antigen constitutively expressed and therefore accessible to the immune system all the time, which would have deleterious consequences.

Proliferation of PBMC of AE patients was seen in response to all recombinant self-antigens in sensitized patients (data not shown), indicating an involvement of autoantigen-specific T cells in the pathogenesis of AE.

Taken together, the ability of all five selected recombinant proteins to bind serum IgE, activate basophils, and induce the proliferation of PBMC of sensitized individuals demonstrates a high specificity of the high-throughput screening technology. Based on this fact, the majority, if not all, of the sequences presented (Table 5 in the Online Repository) probably encode IgE-binding self-antigens, indicating a broad repertoire of human proteins involved in the pathogenesis of AE.

Like other complex diseases, AE is likely to be determined by many genetic factors interacting with environmental components (206). It is therefore not trivial to assess the contribution of each single determinant to the complex phenotype of AE. However, there is cumulating evidence that IgE-mediated autoreactivity to self-antigens can contribute to the exacerbation of eczematous reactions. The demonstration that IgE autoantibodies target keratinocytes in AE patients (204), that self-antigens can cause disintegration of respiratory epithelial cell layers and apoptosis of skin keratinocytes (179), and induce eczematous reactions in healthy skin areas of AE patients in atopy patch tests (81) provide clear evidence for an involvement of autoreactivity in the perpetuation of AE. Based on our results derived exclusively from adult patients we cannot answer the questions of how early the onset of IgE-mediated autoreactivity to self-antigens occurs or if such a sensitization can occur in absence of sensitization to environmental allergens.

These important questions will require investigation of a pediatric population in a following study.

Of course avoidance of exposure is not an option for AE patients sensitized to self-antigens. However, because IgE-mediated reactivity to self-antigens seems to be confined to patients with chronic long lasting AE (178, 169), determination of self-antigen-specific IgE might open new ways for stratification of patients. Patients with IgE-mediated autoreactivity might therefore benefit from systemic therapeutic options like treatment with immunosuppressive agents, which should be limited to severe refractory AE cases (207). The availability of a long list of highly pure recombinant IgE-binding self-antigens builds a solid basis for future investigations aimed at clarifying the role of autoreactivity in the pathogenesis of AE.

Online repository

patient	sex	age [years]	SCORAD	total IgE [kU/l]	self-antigen-specific IgE to*						
					1	2	3	4	5	6	7
1	f	37	14.30	44.60	+	-	-	+	+	-	-
2	f	22	18.25	31.10	+	+	+	+	+	-	-
3	f	24	18.50	15.20	-	-	-	-	+	-	-
4	f	23	16.00	97.60	-	-	+	-	-	+	+
5	f	43	31.70	92.70	-	+	+	-	-	-	-
6	f	23	46.80	22.60	-	-	-	-	+	-	-
7	f	25	47.50	97.00	-	+	+	-	+	-	-
8	m	28	61.04	18.50	-	+	-	-	-	-	-
9	f	20	31.30	99.40	+	-	-	+	+	-	-
10	f	16	16.30	11.40	-	-	-	-	+	-	-
11	m	27	54.60	95.70	-	-	-	-	-	-	+
12	f	27	22.40	6.43	-	-	+	-	+	-	-
13	f	27	37.50	28.70	-	-	-	-	+	-	-
14	m	19	85.20	50.70	-	-	-	-	-	-	-
15	m	42	60.00	2.94	-	-	-	-	-	-	-
16	m	67	38.0	94.20	-	-	-	-	-	-	-
17	m	46	7.50	35.40	-	-	-	-	-	-	-
18	f	34	35.00	66.40	-	-	-	-	-	-	-

Table 4. Characteristics of non-atopic eczema patients with and without autoreactive IgE. Self-antigen-specific IgE to human actin-α (1), tubulin-α (2), eIF6 (3), HLA-DR-α (4), RP1 (5), cyclophilin B (6), and thioredoxin (7) of non-atopic eczema patients are shown.

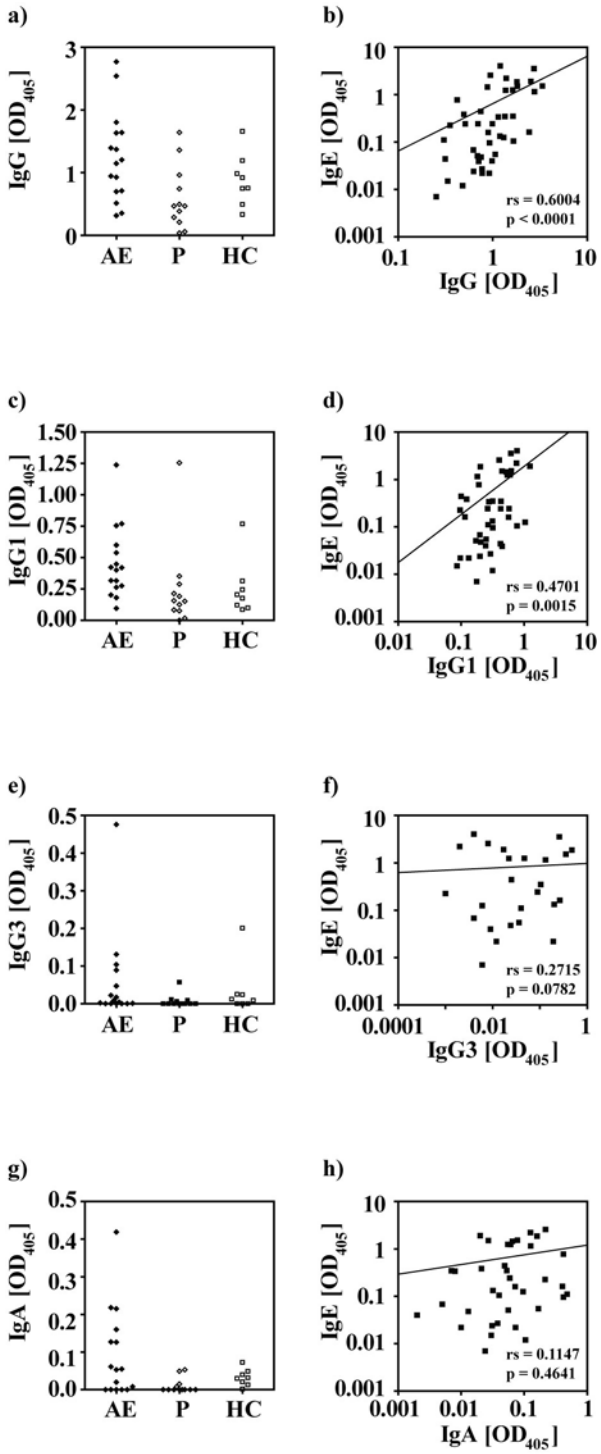

Figure 10. IgG, IgG1, IgG3, and IgA specific for eIF6. Levels of IgG (a), IgG1 (c), IgG3 (e), and IgA (g) specific for recombinant eIF6 were analyzed by ELISA in sera from 16 AE-patients (AE), 12 psoriasis patients (P), and 8 healthy controls (HC). The correlation with eIF6-specific IgE levels is shown (b, d, f, h). Spearman's rank correlation coefficients were calculated.

Clone	ORF[a)]	Database match gene	Gene bank accession no.	Amino acids[b)]	Sequ. contig (bp)	Clone frequ.	Ref[c)]
A) Human sequences described as self-antigen:							
R2b16	C	SOD2 (manganese superoxide dismutase)	NM_000636	198	657	1294	132
P_2	C	RPLP2 (ribosomal P_2 protein)	NM_001004	115	460	892	169
Profilin	C	PFN1 (profilin 1)	NM_005022	140	793	156	166
Cyp B	C	PPIB (cyclophilin B)	NM_000942	216	893	238	172
Cyp A	C	PPIA (cyclophilin A)	NM_021130	165	703	5	172
Cyp C	C	PPIC (cyclophilin C)	NM_000943	212	1058	4	172
TRX	C	TXN (thioredoxin)	NM_003329	105	412	706	82
R3j17	P	alpha-NAC	AJ278883	215	348	1	178
P1m01	P	KRT6A (cytokeratin II)	NM_005554	564	456	1	178
P7n01	P	RPL3 (ribosomal protein L3)	NM_000967	403	497	1	214
R2n23	P	HSP90AA1	NM_005348	732	836	12	168
P6j22	P	ACTA2 (actin alpha 2)	NM_001613	377	608	1	d)
S1c02	P	TUBA1a (tubulin alpha 1a)	NM_006009	451	602	237	d, 213)
P6j13	P	EIF6 (translation initiation factor 6)	NM_002212	245	745	1	d)
R2k20	P	HLA-DR alpha	NM_019111	254	704	291	d)
S2h03	P	MAPRE2 (RP1)	NM_014268	327	820	1	d)
B) New human sequences homologous to environmental allergens:							
P3j02	P	CALM2 (calmodulin 2)	NM_001743	149	584	1	201
R1o01	P	MYO1C (myosin 1C)	NM_033375	1028	470	270	215
P7a22	P	TGM2 (transglutaminase 2)	NM_004613	687	872	1	203
C) Described human proteins:							
P2h18	P	CYP4B1 (cytochrome P450)	NM_001099772	512	484	1	e)
R3i16	P	FLNB (filamin B)	NM_001457	2602	965	474	
r2e02	P	RPL4 (ribosomal L4 protein)	NM_000968	427	974	192	
P4l04	P	ACTL6A (actin-like protein 6A)	NM_178042	387	807	1	
P5m06	P	HIP1R (huntingtin interacting protein 1 related)	NM_003959	1068	858	1	
P4n01	P	PPT1 (palmitoyl-protein thioesterase 1)	NM_000310	306	795	1	
R6d05	P	HBEGF (heparin-binding EGF-like growth factor)	NM_001945	208	775	1	
R2c14	P	VPS24 (vacuolar protein sorting 24 homolog)	NM_016079	222	769	1	
S1e18	P	TPP1 (tripeptidyl peptidase I)	NM_000391	563	868	1	
R3l05	P	EFNA1 (ephrin-A1)	NM_004428	205	917	81	

S1a09	P	PSAP (prosaposin)	NM_001042466	526	989	14
R3i23	P	SFTPA2B (surfactant, pulmonary-associated protein A2B)	NM_006926	248	1169	1
P1b23	C	SNRCP (small nuclear ribonucleoprotein polypeptide C)	NM_003093	159	690	1
S5m03	P	SAT1 (spermine N1-acetyltransferase 1)	NM_002970	171	1014	1
P2j04	P	TIMP3 (TIMP metallopeptidase inhibitor 3)	NM_000362	211	697	1
P3j20	P	RHBDD2 (rhomboid domain containing protein 2)	NM_001040457	223	714	1
R3d03	P	CHPT1 (choline phosphotransferase 1)	NM_020244	406	680	1
S3h17	P	LRRC36 (leucine rich repeat containing protein 36)	NM_018296	754	664	1
S6g04	P	P4HA2 (proline 4-hydroxylase)	NM_001017973	533	737	1
S1j21	P	STK 10 (serine/threonine kinase 10)	NM_005990	968	641	49
S5k16	P	HTATIP2 (HIV-1 Tat interactive protein 2)	NM_001098520	276	893	1
P1n01	P	MTMR14 (myotubularin related protein 14)	NM_001077525	650	888	1
P5g20	P	TRIM26 (tripartite motif-containing protein 26)	NM_003449	539	583	1
P1j19	P	PSMA3 (proteasome subunit alpha 3)	NM_002788	255	612	1
S1p02	P	LGALS3 (galectin 3)	NM_002306	250	770	1
P5c14	P	STAB 1 (stabilin 1)	NM_015136	2570	580	1
S6e22	P	GAB2 (GRB2-associated binding prot. 2)	NM_080491	676	686	1
S3e8	P	PCK2 (phosphoenolpyruvate carboxykinase 2)	NM_004563	640	596	1
P1m24	P	BSG 1 (basigin 1)	NM_001728	385	589	1
P6c12	P	GABARAP (GABA receptor-protein)	NM_007278	117	575	1
P1h09	P	H3F3B (H3 histone 3B)	NM_005324	136	588	1
S7l09	P	ZHX3 (zink fingers and homeoboxes 3)	NM_015035	956	580	1
S5o05	P	KIF13B (kinesin family member 13)	NM_015254	1826	671	1
S7f08	P	UNC119B (unc-119 homolog B)	NM_001080533	251	568	1
P4g24	C	HSPB1 (Heat shock 27 kDa protein 1)	NM_001540	205	790	1
R2m18	P	PIK3R3 (phosphoinositide-3-kinase)	NM_003629	461	570	35
R3l03	P	PSME3 (proteasome activator subunit PA28)	NM_176863	267	557	53

S1e21	P	(COQ9) Coenzyme Q9 homologue	NM_020312	318	527	1
S5a21	P	UBE3C (ubiquitin protein ligase E3C)	NM_014671	1083	596	1
P3n24	P	NCF4 (neutrophil cytosolic factor 4)	NM_000631	339	593	1
S1h20	P	PPL (periplakin)	NM_002705	1756	517	1
P1n15	P	TRAF2 (TNF receptor-associated factor 2)	NM_021138	501	522	1
P7d18	P	MBTPS1 (membrane-bound transcription factor peptidase)	NM_003791	1052	550	1
S4m22	P	ARF1 (ADP-ribosylation factor 1)	NM_001024228	181	690	1
P1d09	P	DUSP6 (Dual specificity phosphatase 6)	NM_001946	381	802	1
R2d06	P	EIF4E2 (translation initiation factor 4E2)	NM_004846	245	563	1419
R1n21	P	GTF2I (general transcriptiopn factor II)	NM_032999	998	483	1
R4b22	P	CAPNS1 (calpain)	NM_001749	268	482	1
P6h03	P	JOSD1 (Josephin domain containing protein 1)	NM_014876	202	579	1
S1g15	P	B4GALT5 (beta 1,4-galactosyltransferase)	NM_004776	388	506	1
P1g21	P	DGKG (diacylglycerol kinase gamma)	NM_001080744	766	471	1
P3m15	P	DAP3 (death associated protein 3)	NM_033657	398	654	1
S5c06	P	UBN1 (ubinuclein 1)	NM_001079514	1134	529	1
P4g19	P	CFL1 (cofilin 1)	NM_005507	166	487	1
P6d22	P	BCAP31 (B-cell receptor-associated protein 31)	NM_005745	246	432	1
R1a01	P	SYK (spleen tyrosine kinase)	NM_003177	635	837	28
R2i03	P	BGN (biglycan)	NM_001711	368	423	192
P1h16	P	VSIG4 (V-set and immunoglobulin domain containing 4)	NM_001100431	305	577	1
S3d08	P	SLIT2 (slit homolog 2)	NM_004787	1529	778	1
S4h08	P	CCNDBP1 (cyclin D- binding-protein 1)	NM_012142	360	811	1
R3d02	P	SERPING1 (serpin peptidase inhibitor)	NM_001032295	500	379	1
R4k08	P	MPG (methylpurine DNA glycosylase)	NM_002434	298	429	1
P6b04	P	TOB2 (transducer of ERBB2)	NM_016272	344	550	1
P3a10	P	LAMC2 (laminin gamma 2)	NM_005562	1193	559	1
R3c07	P	BMP4 (bone morphogenetic protein 4)	NM_130851	408	420	101
S2c07	P	ARF3 (ADP-ribosylation factor 3)	NM_001659	181	549	1
P5g08	P	TPP2 (tripeptidyl peptidase II)	NM_003291	1249	368	1

R2h18	P	PKIG 3 (protein kinase inhibitor gamma)	NM_181804	76	439	324
P1i02	P	SLC22A18 (carrier family 22, memb. 18)	NM_002555	424	300	81
P3h08	P	ACTB (actin beta)	NM_001101	375	781	1
S3n19	P	TMEM116 (transmembrane protein 116)	NM_138341	245	1083	1
S1m01	P	MKNK2 (MAP kinase interacting serine/threonine kinase 2)	NM_199054	465	530	285
P5d12	P	DEDD (death effector domain containing protein)	NM_001039712	318	693	1
P2j17	P	JUNB (jun B proto-oncogene)	NM_002229	347	534	1
R3g23	P	MED27 (mediator complex subunit 27)	NM_004269	311	633	70
R2e7	P	(MMRN2) multimerin 2	NM_024756	949	813	1
P7l04	P	HGSNAT (heparan-alpha-glucosaminide N-acetyltransferase)	NM_152419	635	450	1
P6b07	P	PBX2 (pre-B-cell leukemia homeobox 2)	NM_002586	430	420	1
R4m17	P	FXYD6 (FXYD domain containing ion transport regulator 6)	NM_022003	95	662	1
P2i12	P	BSPRY (B-box and SPRY domain containing protein)	NM_017688	402	566	1
R2f15	P	CPNE2 (copine II)	NM_152727	548	606	69
R1c02	P	MIF4GD (MIF4G domain containing)	NM_020679	256	351	1
P1a01	P	ATP13A1 (ATPase 13A1)	NM_020410	1086	446	1
P3o13	P	TRIM47 (tripartite motif-containing 47)	NM_033452	638	550	1
R2e21	P	ANXA11 (anexin A 11)	NM_145869	505	686	127
R2o04	P	ADAMTSL4 (ADAMTS-like 4)	NM_019032	1074	814	23

D) Predicted proteins:

S6o18	P	RNF187 (ring finger Protein 187)	XM_928029	403	648	1
R3c20	P	LOC728054	XM_001128749	91	426	97
P7o22	P	LOC400927	NR_002821	148	511	1
S4i16	?	LLNLR-294C8	AC099491	?	587	1
P4f02	P	Chromosome 1 ORF 158	NM_152290	194	661	1
P5m13	P	Chromosome 14 ORF1	NM_007176	140	434	1
R1m01	P	Chromosome 20 ORF3	NM_020531	416	573	53

E) Chromosomal contigs:

P6k24		Mitochondrion complete genome	NC_001807		290	1
P7n09		Chromosome 1 genomic contig	NT_004321		509	1

P1o09	Chromosome 1 genomic contig	NT_032977	460	1
R4o22	Chromosome 1 genomic contig	NW_921351	579	1
R4c19	Chromosome 2 genomic contig	NT_022184	589	1
P3c06	Chromosome 2 genomic contig	NW_927808	550	1
P4n22	Chromosome 2 genomic contig	NW_921618	520	1
R1f20	Chromosome 3 genomic contig	NT_029928	493	1
S7b11	Chromosome 4 genomic contig	NT_022792	463	1
S1h06	Chromosome 5 genomic contig	NT_006713	772	1
S2j13	Chromosome 5 genomic contig	NT_006576	551	1
S2o02	Chromosome 6 genomic contig	NT_010498	553	1
S1c20	Chromosome 7 genomic contig	NT_007819	841	1
P3g21	Chromosome 7 genomic contig	NT_007758	511	1
S1i04	Chromosome 9 genomic contig	NT_008470	614	1
R3n23	Chromosome 10 genomic contig	NT_008583	637	1
P4j23	Chromosome 11 genomic contig	NT_033899	670	1
R4i06	Chromosome 11 genomic contig	NT_033927	602	1
S4n06	Chromosome 11 genomic contig	NT_033903	544	1
R1e03	Chromosome 12 genomic conig	NT_009714	506	1
R1i02	Chromosome 13 genomic contig	NT_009952	1013	1
R2i19	Chromosome 13 genomic contig	NT_024524	413	1
R3e20	Chromosome 14 genomic contig	NT_026437	489	1
R6g13	Chromosome 15 genomic contig	NT_010274	903	1
R1j3	Chromosome 15 genomic contig	NT_010194	948	1
R2g09	Chromosome 16 genomic contig	NW_926140	893	1
S5n15	Chromosome 22 genomic contig	NT_011519	489	1
R2p09	Chromosome X genomic contig	NT_011651	697	1

F) Clones with very short inserts (1-4 amino acids): 10

G) Empty clones: 72

H) Contaminants from other libraries: 1

Table 5: Characteristics of IgE-binding sequences.

a) Predicted open reading frame of the isolated inserts: C, complete; P, partial.

b) Number of amino acids predicted from the complete reading frames

c) Reference describing the original environmental allergen

d) This work

e) Cytochrome C is described as allergen from fungi (Cur l 3) and grasses (Lol p 10, Poa p 10), however these proteins are not members of the cytochrome P450 protein family.

7.2 The IgE-binding self-antigens tubulin-α and HLA-DR-α are overexpressed in the lesional skin of atopic eczema patients

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Short communication

Submitted to Allergy

Abstract

Background: Atopic eczema is the most common chronic, relapsing, inflammatory skin disorder with an atopic background. Previous studies have shown that IgE-mediated reactivity to self-antigens plays a role in the pathogenesis of the disease. However, the expression of self-antigens associated with atopic eczema in the lesional skin is poorly investigated.

Aim of the study: This study was aimed to show that IgE-binding self-antigens are overexpressed in atopic eczema lesions.

Methods: Tubulin-α and HLA-DR-α, two recently described self-antigens, were stained by immunohistochemistry in skin specimens from chronic and acute atopic eczema lesions, unaffected skin from the same patients or skin from healthy controls.

Results: The expression of tubulin-α and HLA-DR-α is upregulated in atopic eczema lesions compared to non-lesional or healthy skin and correlates with the number of infiltrating immune cells and the degree of inflammation.

Conclusion: Upregulation of IgE-binding self-antigens in lesional skin of atopic eczema patients might further promote the existing inflammation and induce exacerbations of the disease in the absence of exposure to environmental allergens.

Introduction

Atopic eczema (AE) is the most common inflammatory skin disorder affecting up to 10-20% of children and 1-3% of adults in industrialized countries (59). Multiple factors are involved in the pathogenesis of AE including genetic predisposition, impaired skin barrier function, microbial colonization, and sensitization against environmental allergens. In addition, IgE antibodies reacting with human self-antigens are supposed to be involved in the pathogenesis of the disease (83).

Several IgE-binding self-antigens associated with AE were identified, such as profilin, ribosomal protein P_2, manganese superoxide dismutase (MnSOD), cyclophilin, thioredoxin, and Hom s 1-5 (For a review see (83)). By screening a human cDNA library displayed on phage surface with immobilized serum IgE from AE patients we recently identified 140 additional IgE-binding self-antigens (208), demonstrating that a broad spectrum of IgE-binding self-antigens is associated with AE. Recombinant human self-antigens characterized in detail were shown to bind serum IgE of AE patients, to induce mediator release from basophils, and to stimulate the proliferation of PBMC (166, 132, 169, 82, 178). Moreover, serum IgE of AE patients targets keratinocytes and normal human epidermis (204) and the well-characterized self-antigen MnSOD is sufficient to elicit eczematous reactions if applied to healthy skin areas of AE patients (81). Interestingly, MnSOD expression is upregulated in lesional, but not in healthy skin areas of AE patients sensitized to the self-antigen, providing strong evidence for the involvement of autoreactivity in the exacerbation of an existing inflammation (81).

During the present study we investigated the expression of two newly described self-antigens, tubulin-α and HLA-DR-α (208), by immunohistochemistry in skin biopsies taken from acute and chronic AE lesions and non-lesional skin of the same patients, or from healthy controls.

Methods

Patient selection

Biopsies were taken from four AE patients and three healthy controls of both sexes. Diagnosis of AE was made according to the recommendations of the EAACI and severity of the disease was determined by the SCORAD index as described (81). Total and specific serum IgE against *Malassezia sympodialis* were determined by the ImmunoCap system (Phadia, Uppsala, Sweden). Healthy individuals had no history of allergy, asthma, or AE and normal total IgE levels. The characteristics of patients and controls are reported in Table 6. Permission to conduct this study was obtained from the Ethics Committee at Karolinska Hospital, Stockholm, and informed consent was given by all subjects.

donor	sex	age (y)	SCORAD	Phadiatop	total IgE (kU/l)	IgE to *Malassezia* spp. (m70)
AE 1	m	49	48	pos	2700	24
AE 2	f	20	48	pos	2300	28
AE 3	m	43	61	pos	1700	7.5
AE 4	f	26	53	neg	81	<0.35
HC 1	m	41	-	neg	77	<0.35
HC 2	f	23	-	neg	11	<0.35
HC 3	f	23	-	neg	6.2	<0.35

Table 6. Characteristics of patients and healthy controls. AE, atopic eczema; HC, healthy control; SCORAD, severity scoring of atopic dermatitis

Immunohistochemistry staining

Immunohistochemistry staining was performed on cryostat-embedded skin specimens taken from lesional and non-lesional skin of AE patients or of healthy controls. Frozen sections were fixed in 50% and 100% acetone and incubated in 0.3% H_2O_2 in PBS to inhibit endogenous peroxidase activity. Non-specific binding was blocked with normal horse sera diluted 1:10 in 4% BSA, followed by incubation with avidin and biotin blocking solutions (Vector Laboratories, Burlingame, US). Human tubulin-α, HLA-DR-α, and CD3 were detected with specific primary antibodies: mouse anti-human tubulin-α, clone TU-01 (AbD Serotec, Kidlington, UK) diluted 1:250; mouse anti-human HLA-DR-α, clone TAL.1B5 (Dako, Glostrup, Denmark) diluted 1:12.5; mouse anti-human CD3, clone SK7

(BD Biosciences, Franklin Lakes, US) diluted 1:16. Mouse IgG1 (Dako) was used as isotype control. After incubation with biotinylated horse anti-mouse IgG secondary antibody (Vector Laboratories) diluted 1:10, biopsies were developed with ABC-elite solution (Vector Laboratories) and 3-amino-9-ethylcarbazole (Sigma, St. Louis, US) as substrate. All slides were counterstained with Mayer's hematoxylin and examined by standard bright field optics.

Results

Human tubulin-α and HLA-DR-α were stained by immunohistochemistry in skin biopsies taken from chronic lesions, positive APT reactions and non-affected skin of four AE patients or from healthy skin of three non-atopic individuals.

A strong upregulation of tubulin-α expression was found in epidermal cells of chronic (Figure 11A) and acute AE lesions of all four patients tested. In contrast, tubulin-α expression was strongly reduced in keratinocytes from non-affected skin of the same patients (Figure 11B) and confined to stratum granulosum in the skin of the healthy individuals (Figure 11C).

HLA-DR-α was highly expressed on infiltrating cells such as B cells, dermal dendritic cells, and Langerhans cells in chronic AE lesions (Figure 11D) and positive APT reactions (data not shown). In biopsies taken from non-affected skin of the same AE patient (Figure 11E) or from healthy donors (Figure 11F) HLA-DR-α expression was strongly reduced compared to inflamed skin. Moreover, the degree of HLA-DR-α staining correlated quite well with the degree of inflammation, determined by the numbers of infiltrating $CD3^+$ T cells (Figure 11G) whereas only few $CD3^+$ T cells were detectable in non-lesional skin of AE patients or in skin of healthy individuals (Figure 11H, I). Staining of the skin with mouse IgG1 used as isotype control were negative in all biopsies tested and independent from inflammatory processes as expected (Figure 11J-L).

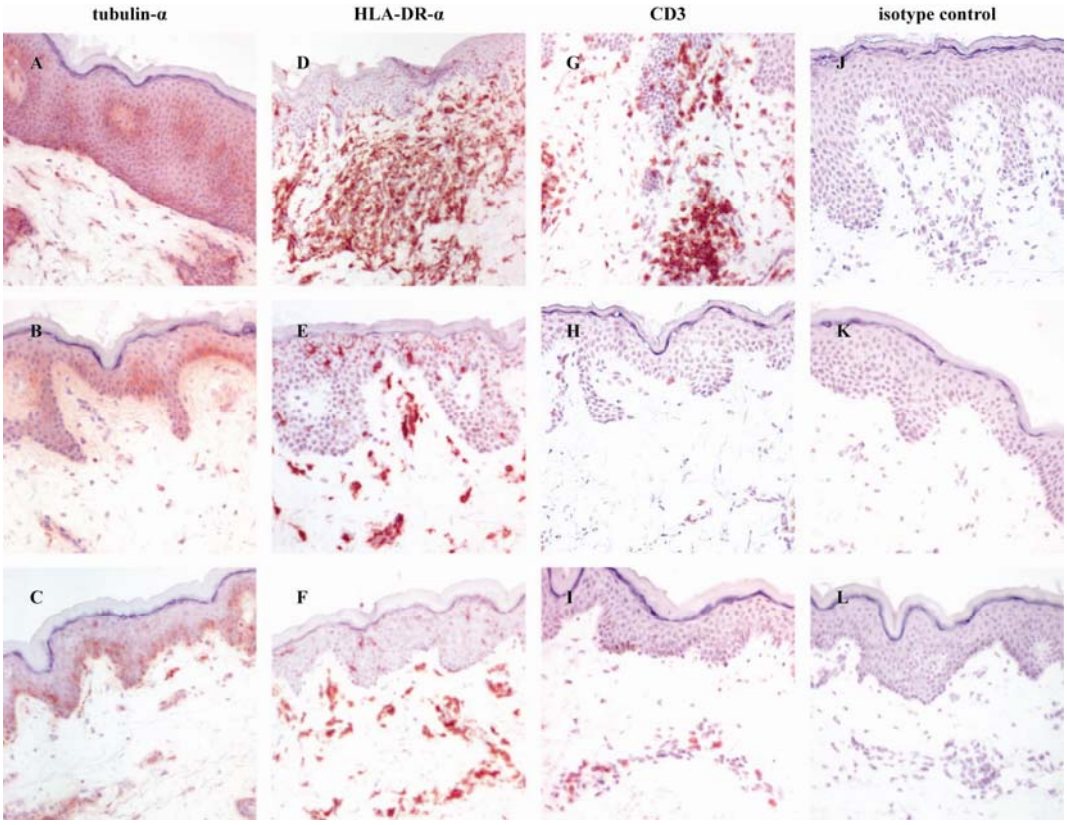

Figure 11. Upregulation of tubulin-α and HLA-DR-α expression in AE lesions. Tubulin-α and HLA-DR-α were stained by immunohistochemistry in the lesional (A, D), non-lesional skin (B, E) of the same AE patients and in healthy skin (C, F). Infiltrating $CD3^+$ cells (G, H, J) reflect the degree of inflammation. Results obtained for one representative AE patient and one healthy control are shown. Isotype control staining for lesional skin (J), non-lesional skin of the same patient (K), and skin of a healthy individual (L) is shown.

Discussion

IgE-mediated autoreactivity is assumed to play a role in the multifactorial pathogenesis of AE and a broad spectrum of IgE-binding self-antigens has been described (83). They include proteins with a high degree of homology to environmental allergens (166, 132, 169, 82, 80) as well as self-antigens without any sequence homology to known allergens (178). Autoreactivity to self-antigens sharing sequence homology to environmental allergens can be explained by molecular mimicry as clearly shown for MnSOD (132, 81), cyclophilin (80), and thioredoxin (82). These proteins are inducible by oxidative stress (209), a condition characteristic for inflamed skin of AE patients (210), and in fact it has been shown that MnSOD is moderately expressed in the skin of healthy individuals or in healthy skin areas of AE patients, but strongly upregulated in lesional skin areas (81). Moreover, application of human MnSOD to unaffected skin areas of AE patients in atopy patch tests is sufficient to elicit eczematous reactions in AE patients sensitized to MnSOD, highlighting the role of IgE-mediated autoreactivity in the exacerbation and/or perpetuation of AE (81). However, overexpression of other IgE-binding self-antigens in the inflammatory skin areas of AE patients has not been reported. During the present study we analyzed the expression of two IgE-binding self-antigens, tubulin-α and HLA-DR-α, in lesional and non-affected skin of AE patients and in the skin of healthy individuals by immunohistochemistry. Both, tubulin-α and HLA-DR-α are detectable in epidermal keratinocytes and infiltrating immune cells, respectively. The expression of the self-antigens is upregulated in skin specimens taken from chronic AE lesions compared to unaffected skin of the same patients or skin from healthy individuals (Figure 11). These findings corroborate previous work, demonstrating that the IgE-binding self-antigen MnSOD is upregulated in inflamed skin of AE patients. The overexpression of self-antigens in AE lesions provides targets for autoreactive serum IgE antibodies at the site of inflammation allowing the formation of IgE immune complexes that could target effector cells like mast cells and basophils. Clear evidences supporting this assumption are the ability of IgE-binding self-antigens to induce mediator release from basophils (208) and immediate type I skin reactions (132, 169, 82, 178, 80). Activation of effector cells results in the release of preformed mediators, production of cytokines, and initiation of an allergic tissue reaction, resulting in induction and maintenance of inflammatory skin responses (179). In summary, overexpression of IgE-binding self-antigens in lesional skin of AE patients seems to be a common phenomenon, which can explain the significant correlation observed between autoreactivity and severity of the disease (211). Since a relevant subset of about 30% AE patients shows IgE reactivity to a variety of human self-antigens, it is likely that these reactions contribute to the exacerbation of AE in a subset of patients.

8 Final Discussion

Characterized by typically distributed eczematous skin lesions, dry skin, and pruritus, AE severely impairs the quality of patients' life. In addition, AE represents the most common chronic inflammatory skin disease and its prevalence still increases, causing rising health cost burdens to the society. To date allergen-specific immunotherapy is the only treatment to cure allergic diseases and is successfully used in clinical practice for the treatment of relatively simple allergic complications like insect venom allergy and allergic rhinitis. However, most of the clinical trials aimed to treat complex allergic disorders such as AE, or food allergy were inconclusive. To facilitate the management of AE and to define new therapeutic strategies, a detailed understanding of the molecular components involved in the development of AE and of the immunological mechanisms underlying the pathogenesis of the disease are required.

Already in the 1940s, it was assumed that autoreactivity could play a role in the pathogenesis of AE since human skin dander elicits type I skin reactions in those patients. This concept was not followed further until the use of modern molecular biology methods allowed identification, cloning, and production of IgE-binding self-antigens. Detailed characterization of the IgE-binding self-antigens demonstrates that some human proteins can cross-react with environmental allergens due to shared features on primary and secondary structure levels. Some highly conserved IgE-binding self-antigens associated with AE such as MnSOD, cyclophilin, and thioredoxin were characterized in deep molecular detail *in vitro* and tested for skin test reactivity *in vivo*. In these cases cross-reactivity can be traced back to shared structural components between self-antigens and environmental allergens. In addition, screening of a cDNA library constructed from a human epithelial cell line with serum IgE from AE-patients led to the identification of five additional IgE-binding self-antigens, designed Hom s 1-5. Out of these, Hom s 1-3 and Hom s 5 do not show homology to any known environmental allergen.

Although many autoreactive self-antigens are described, the spectrum of IgE-binding self-antigens is supposed to be far from being complete. Thus, the major aim of the present thesis was to estimate the extent of the IgE-binding self-antigen repertoire associated with AE.

8.1 Identification of a broad spectrum of self-antigens associated with atopic eczema

A human cDNA library displayed on phage surface was constructed and screened with serum IgE from AE-patients by high-throughput technologies. Sequencing of the enriched clones resulted in 140 diverse sequences.

Among them, ten already described self-antigens were present, indicating a high specificity of the screening procedure. Five sequences showed homology to known environmental allergens, namely tubulin-α, ribosomal protein L3, calmodulin, myosin, and transglutaminase from storage mites (212, 213), *A. fumigatus* (214), pollen (128), *Blattella germanica* (www.allergome.org (215)), and *Dermatophagoides farinae* (203), respectively. Considering previous results on the IgE-binding capacity of conserved self-antigens (171, 80, 82), human tubulin-α, ribosomal protein L3, calmodulin, myosin, and transglutaminase are supposed to cross-react with the corresponding environmental allergens. A large group of 90 sequences coded for human proteins without sequence homology to described environmental allergens. Seven sequences encoded putative proteins, 27 sequences matched to chromosomal contigs derived from different chromosomes, and one sequence matched to mitochondrial DNA.

8.2 Production of recombinant self-antigens

Out of the group of 90 sequences encoding human proteins without homology to known environmental allergens five sequences were randomly selected and together with the known self-antigens cyclophilin B and thioredoxin immunologically characterized in detail. The genes for actin-α, tubulin-α, eukaryotic initiation factor for translation 6 (eIF6), HLA-DR-α, and RP1 were amplified by PCR from a human cDNA library using gene-specific primers and cloned as His_6-tagged fusion proteins into a high level expression vector. The selected proteins were recombinantly produced in *E. coli* and purified by IMAC. A desalting step prior to IMAC reduced bacterial contaminations such as lipopolysaccharide (LPS). Highly pure protein solutions with concentrations in the range between 0.5-1.2 mg/ml and LPS concentrations of 0.1-0.2 EU/µg protein were obtained for further investigations.

8.3 Immunological characterization of recombinant self-antigens

Western blot experiments using sera of AE-patients confirmed the IgE-binding capacity of the recombinant self-antigens. The prevalence of sensitization among AE-patients was determined by ELISA and found to be 15.5%, 21.7%, 25.4%, 8.7%, and 29.0% for actin-α, tubulin-α, eIF6, HLA-DR-α, and RP1, respectively. 9.2% of AE-patients had specific IgE against cyclophilin and 23.4% showed IgE-binding to thioredoxin. In summary, 51 out of 71 (71.8%) AE-patients had autoreactive IgE-antibodies to at least one of the tested self-antigens, while none of the healthy controls showed detectable levels of self-antigen-specific IgE. These findings are in good agreement with previously reported frequencies of prevalence for other self-antigens (81, 204). Since all randomly chosen proteins bound serum IgE, the used screening system is suitable for an efficient isolation of allergens with affinity to IgE and most likely, all 140 newly identified sequences encode IgE-binding self-antigens.

The mean age, the sex ratio, and the severity of the disease determined by the SCORAD index were similar among the groups of patients with and without autoreactive IgE. In contrast, total IgE values were significantly higher among the subset of patients showing IgE-mediated autoreactivity. 13 and five patients with and without autoreactive IgE, respectively, suffered from non-atopic eczema, defined by low total IgE-levels (<150 kU/l) and lack of sensitization to environmental allergens in routine assessments (60).

Tubulin-α is described as allergen from the storage mites *Tyrophagus putrescentiae* (212) and *Lepidoglyphus destructor* (213). Both proteins share 94% identity with the human homologue. Inhibition experiments showed that human tubulin-α in the liquid phase is able to inhibit the binding of serum IgE to tubulin-α from *Lepidoglyphus destructor* coated onto the solid phase in ELISA. Thus, human and mite tubulin-α are cross-reactive due to common structural features and shared IgE-binding epitopes (data not shown).

Next to specific IgE the antibody-isotypes IgA, IgG, IgG1, IgG3, and IgG4 specific for eIF6 were analyzed. AE-patients had elevated levels of specific IgA and IgG4 compared to healthy controls. In contrast, the levels of total specific IgG, specific IgG1, and IgG3 were similar between AE-patients and healthy individuals. These findings corroborate previous reports that demonstrated the presence of IgG-antibodies specific for the *A. fumigatus* allergens in sera from healthy and allergic individuals (196, 195).

AE is characterized by severe pruritus that is mainly due to the release of histamine from activated basophils and mast cells. Except HLA-DR-α all tested self-antigens were able to cross-link FcεR-bound IgE on the surface of basophils and to induce the release of

mediators, accompanied by the expression of CD63 (216, 198) and the upregulation of CD203c (197, 217). The failure of HLA-DR-α to cross-link FcεR might by due to only one IgE-binding epitope, resulting in monovalent IgE-binding. HLA-DR-α is exposed on the outer surface of professional antigen presenting cells and constantly accessible for the immune system. Thus, monovalent IgE-binding would prevent FcεR cross-linking and degranulation of basophils and mast cells, resulting in the absence of symptoms.

Since T effector cells play an important role in the pathogenesis of AE by activating B cells to produce IgE, promoting inflammation in the skin, and inducing keratinocyte apoptosis, the presence of self-antigen-specific T cells was analyzed. Stimulation of PBMC of sensitized individuals with recombinant self-antigens resulted in dose-dependent proliferation of the cells, while cells of non-sensitized individuals or healthy controls did not proliferate. LPS concentrations 100-fold higher than those present in the recombinant protein solutions used were not able to induce proliferation of PBMC, indicating that the observed proliferation is self-antigen-specific (data not shown).

In summary, most if not all of the 140 human proteins identified during this work might serve as an IgE-binding self-antigen in AE since 1) a large number of potential IgE-binding self-antigens associated with AE were identified by phage display techniques, 2) the IgE-binding capacity of five randomly selected self-antigens was verified by Western blot, ELISA, and basophils activation assays, and 3) the identified potential self-antigens share no obvious common feature. Moreover, 71.8% of the analyzed patients show IgE-mediated autoreactivity against at least one out of five randomly selected self-antigens. Assuming that a long list of IgE-binding self-antigens associated with AE exists, most or even every AE patient might have autoreactive IgE-antibodies in serum.

In general, several mechanisms may induce autoreactivity, including viral or bacterial superantigens, bystander activation of autoreactive T cells by cytokines secreted in response to viral or bacterial pathogens (218), release of self-antigens during inflammation, and molecular mimicry.
More than 90% of AE patients show colonization of the skin by the Gram-positive bacteria *S. aureus*, whose superantigenic enterotoxins activate T cells in an allergen-independent manner. These staphylococcal enterotoxins were shown to stimulate autoreactive T cells and induce exacerbations in experimental autoimmune encephalitis (EAE) mouse model of multiple sclerosis (219). In addition, environmental allergens activate various immune cells in AE, resulting in the release of cytokines and chemokines that may decrease the threshold for activation of autoreactive T cells (220, 221). The dermal inflammation

together with scratching leads to the damage of human cells and the release of self-antigens, which are not accessible to the immune system under healthy conditions. Indeed, it has been demonstrated that IgE autoantibody levels increase after allergic inflammation and tissue damage (178, 222). Moreover, molecular mimicry is supposed to play a decisive role in inducing autoreactivity in AE since infections with pathogens, such as *M. sympodialis* expressing antigenic homologues of human proteins may promote the activation of potentially autoreactive cells (223). The cross-reactivity between conserved human self-antigens and structurally homologous environmental allergens was shown for tubulin-α. But also microbial peptides with limited sequence homology to human self-antigens are effective activators of autoreactive T cells (224). Several bacterial and viral proteins have been shown to activate autoreactive T cell clones specific for myelin basic protein (MBP), the major self-antigen in multiple sclerosis. Interestingly, most of the stimulating bacterial and viral peptides show no obvious sequence similarity with the cross-reactive peptide from MBP, indicating that the antigenic surfaces rather than sequence similarity are decisive for peptide mimicry (225, 226). Thus, also human proteins without sequence homology to environmental allergens like eIF6 or RP1 might activate autoreactive T cells through molecular mimicry.

8.4 Dermal expression of self-antigens

The expression of actin-α, tubulin-α, and HLA-DR-α in skin biopsies of AE patients and healthy individuals was analyzed by immunohistochemistry. All three self-antigens were detectable in acute lesions, chronic lesions, and unaffected skin of AE patients, and in skin biopsies taken from healthy individuals. Actin-α was found in smooth muscle cells in the dermis such as musculus arrector pili associated with hair follicles and smooth muscle cells surrounding dermal blood vessels. Tubulin-α was mainly expressed in epidermal keratinocytes and the level of tubulin-α expression was increased in the epidermis of AE lesions compared to non-lesional skin of the same patient. The expression of HLA-DR-α on professional antigen-presenting cells such as Langerhans cells and IDEC correlated directly with the degree of dermal inflammation, determined by the number of infiltrating $CD3^+$ T cells and hematoxylin-eosin staining. Thus, increased expression of tubulin-α and HLA-DR-α further promotes IgE-mediated autoreactivity during inflammatory processes.

Incubation of skin biopsies from healthy individuals with sera from AE patients led to binding of serum IgE to keratinocytes, while no IgE-binding was detectable after incubation with sera from healthy individuals. This observation demonstrates that IgE antibodies present in sera from AE patients indeed bind their targets in the skin, strongly

indicating that IgE-mediated autoreactivity plays a role in the pathogenesis of AE (data not shown).

8.5 IgE-mediated Reactivity to Self-Antigens: A Controversial Issue

Recent publications and our own data on IgE-mediated reactivity to self-antigens were critically analyzed and published as Review Article in International Archives of Allergy and Immunology:

Zeller S, Glaser AG, Vilhelmsson M, Rhyner C, Crameri R. Immunoglobulin-E-mediated reactivity to self-antigens: a controversial issue. International Archives of Allergy and Immunology 2008; 145:87-93.

Review

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Immunoglobulin-E-Mediated Reactivity to Self Antigens: A Controversial Issue

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Key Words
Immunoglobulin E autoreactivity · Cross-reactivity · Self antigens · Molecular mimicry

Abstract
Immunoglobulin E (IgE) reactivity to self antigens is well established in vitro by ELISA, inhibition ELISA, Western blot analyses and T cell proliferation experiments. In vivo, IgE-binding self antigens are able to elicit strong type I reactions in sensitized individuals and, in the case of human manganese superoxide dismutase, to elicit eczematous reactions on healthy skin areas of patients suffering from atopic eczema. The reactions against self antigens sharing structural homology with environmental allergens can be plausibly explained by molecular mimicry between common B cell epitopes. For the second class of IgE-binding self antigens without sequence homology to known allergens, it is still unclear if the structures are able to induce a B cell switch to IgE production, or if the reactivity is due to sequence similarity shared with not yet detected environmental allergens. However, in all cases, cross-reactivity is never complete, indicating either a lower affinity of IgE antibodies to self allergens than to the homologous environmental allergens or the presence of additional B cell epitopes on the surface of the environmental allergens, or both. Increasing evidence shows that self allergens could play a decisive role in the exacerbation of long-lasting atopic diseases. However, the only observation supporting a clinical role of IgE-mediated autoreactivity is confined to the fact that IgE levels against self antigens correlate with disease severity.

Introduction

Historically, the first description of 'autoreactivity' goes back to the early last century when the sensitivity of man to human skin dander was demonstrated [1, 2]. At that time, it was of course not possible to identify the molecular nature of the endogenous proteins potentially serving as targets for immunoglobulin E (IgE) (auto)-antibodies. For this reason, the concept that IgE-mediated autoreactivity might play a role in the pathogenesis of allergic diseases was not followed further, until progress in modern molecular biology methods allowed to identify, clone and produce IgE-binding self antigens which are expressed in a variety of human tissues and cell types [3, 4]. To date, a long list of IgE-binding self antigens has been described, and those best investigated are reported in table 1. Although IgE-mediated reactivity to self antigens has been clearly shown in vitro and in vivo [3, 5–12], it is still unclear if and how immune recognition of IgE-reactive autoantigens can contribute to the pathogenesis

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of allergic inflammation. However, there is increasing evidence deriving from structural work comparing pairs of homologous environmental and self antigens [11–14] and from cellular work [15–17] indicating that the observed phenomenon of autoreactivity to self antigens is due to molecular mimicry between shared B cell epitopes situated at the end of the sensitization process.

Allergenicity, Autoreactivity and Autoimmunity

In order to understand complex phenomena like the ability of human manganese superoxide dismutase (MnSOD) to elicit eczema formation after application to the skin of MnSOD-sensitized individuals [9, 18], it is important to precisely define the terms allergenicity, autoreactivity and autoimmunity.

The allergenicity of a protein reflects two properties: (1) the potential to induce allergen-specific IgE antibodies through induction of B cell isotype switches (sensitization), and (2) the potential to induce symptoms by cross-linking of membrane-bound allergen-specific IgE on effector cells, normally reflected by a positive skin test in sensitized individuals. These two intrinsic properties of an allergen are not necessarily linked. In fact, sensitization to Api g 1, the major celery allergen which is structurally related to the major birch pollen allergen Bet v 1 [19], is frequent in Central Europe where birch allergy is common, but not in Southern Europe where birch is absent [20]. Although celery allergy is quite common in the Mediterranean area, sensitization in this geographic area occurs through various celery allergens but not through Api g 1 [20], indicating a low allergenic potential of Api g 1, if any. Cellular work involving T cell lines and T cell clones demonstrated that humoral as well as cellular reactivity to Api g 1 is predominantly based on cross-reactivity with the major birch pollen allergen Bet v 1 [21, 22]. Finally, the recently solved crystal structure of Api g 1 [23] allows a deep analysis of the cross-reactivity between Api g 1 and Bet v 1 confirming, at structural level, the predominant role of cross-reactivity in sensitization to Api g 1, earlier shown by mutational epitope analysis [24]. Together, these data clearly demonstrate that allergens like Api g 1 might elicit symptoms by cross-linking the bound IgE on the surface of mast cells, although they are not able to induce the secretion of allergen-specific IgE antibodies by B cells.

Allergenicity related to the ability of a protein to induce symptoms based on cross-reactivity is a quite common phenomenon in pollen-food syndromes as indicated by the consumption of apples which rarely, if ever, induces IgE antibodies but may elicit allergic symptoms in patients with peach allergy or birch pollinosis [16].

Autoreactivity can be defined as an antigen-antibody response to self antigens and is not necessarily linked to an autoimmune response. Like IgE-mediated responses to pollen-food allergens, autoreactivity can occur between self antigens and structurally related environmental antigens as a result of molecular mimicry which will be discussed in detail below. In contrast, autoimmunity results from the production of autoantibodies raised against self antigens [25, 26].

B cell cross-reactivity, which is important for mast cell triggering, is straightforward, as it concerns only B cell epitopes. In contrast to T cell cross-reactivity which involves linear epitopes, B cell cross-reactivity is exquisitely dependent on conformational features [27]. Unfortunately, misconceptions regarding the term 'epitope' are widespread in the literature, probably generated through different experimental setups, as pointed out earlier by Laver et al. [28]. The only method to determine the complete structure of a B cell epitope is the preparation of a complex of a monoclonal antibody Fab fragment with its antigen, cocrystallization of the complex and determination of its structure resolving those determinants recognized by the complementary determining regions of the antibody on the native protein. All Fab/antigen complexes solved so far by X-ray crystallography reveal discontinuous B cell epitopes, including a recent study on binding and structural characterization of cross-reactive phylogenetically conserved *Plasmodium* proteins [29]. This study also shows that the binding affinity of the monoclonal antibody raised against the apical membrane antigen 1 from *Plasmodium vivax* to the homologous cross-reacting apical membrane antigen 1 antigen from *Plasmodium falciparum* is about 100-fold weaker compared with binding to the cognate antigen. This difference in affinity can be traced back to differences in contacts between the antibody and the homologous antigens.

Unfortunately, the common methods used to investigate cross-reactivity such as ELISA, Western blot analyses and inhibition experiments are not sensitive enough to reveal differences in antigen-antibody affinity which might have profound physiological consequences.

IgE-Binding Self Antigens

The first reported autoantigen able to bind serum IgE from allergic individuals was human profilin [5], a protein structurally related to the birch pollen allergen Bet

Table 1. Self antigens confirmed as IgE-binding molecules in vitro and in skin test challenges in vivo

Autoantigen	Homologous allergens	MW kDa	Function	GenBank Acc. No.	PDB ID	Ref.
SART-1 (Hom s 1)		73	cell cycle arrest and apoptosis	AB006198		48, 49
α-NAC (Hom s 2)		33	intracellular localization of nascent polypeptides, activator of transcription	AJ278883		3, 50
BCL7B (Hom s 3)		20	oncogene	X89985		3
Hom s 4	Cyp c 1 Phl p 7	54		Y17711		3, 51
Cytokeratin type II (Hom s 5)		43	component of the cytoskeleton			3
Profilin	Bet v 2	15	actin polymerization	NM_005022	1pfl	5
Ribosomal P_2	Asp f 8	13	part of the 60 S ribosome	AJ224333		7
MnSOD	Asp f 6 Mala s 11 Hev b 10	26	superoxide dismutase	Y00472	1ABM	13
Cyclophilin B	Asp f 27 Asp f 11 Mala s 6	23	peptidyl-prolyl *cis-trans* isomerase	NM_000942	2CPL	11
Thioredoxin	Mala s 13 Tri a 25 Zea m 25 ZmTRX*h*2	12	oxidoreductase	X77584 NM_003329	1ERU	12

MW = Molecular weight; PDB ID = Protein Data Bank identification.

v 2. It was demonstrated that profilins constitute a wide family of pan-allergens [30]; however, human profilin was not able to induce immediate type I reactions in skin test challenges of allergic individuals sensitized to Bet v 2 [4]. A plausible explanation for this observation could be that the affinity of the cross-reactive IgE antibodies raised against Bet v 2 is not high enough for human profilin to induce mast cell degranulation. In contrast, many other self antigens like MnSOD [6], ribosomal P_2 protein [7], cyclophilin (CyP) [8, 11] and thioredoxin [10, 12], which all show a strong structural similarity to environmental allergens, as well as self antigens without structural similarity to environmental allergens like cytokeratin type II or BCL7B [3] clearly elicit positive results in skin test challenges (table 1). However, the number of IgE-binding self antigens is certainly much larger than those described so far. High throughput screening of a human lung cDNA library displayed on phage surface [31, 32] revealed more than 150 partial and complete cDNA sequences potentially encoding IgE-binding proteins. Although not yet analyzed in detail, some of the cDNA sequences, like those encoding α-tubulin [33] or ribosomal L_3 protein [34], show a high degree of sequence identity and cross-reactivity to environmental allergens [Zeller et al., unpublished results]. In these cases, it makes sense to assume that IgE autoreactivity can be traced back to common structural features shared between environmental and self antigens (see below). However, structural similarity of the environmental allergen should probably not be too similar to the corresponding human antigen as strongly autoreactive B cells can be expected to be deleted from the repertoire [16, 35]. Although the repertoire of allergenic structures is supposed to be limited [36], it is unfortunately far from complete. This unsatisfactory situation does not allow a bioinformatic evaluation of cDNA sequences without homology to known environmental allergens for their cross-reactive potential.

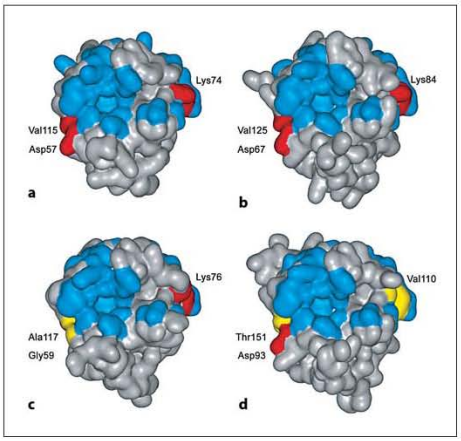

Fig. 1. Solvent exposed surfaces of Mala s 6 and the natural human isoforms CyP B, CyP A and CyP C. Amino acids which are conserved in all structures and exposed to the solvent define two putative IgE-binding epitopes (blue). Asp57 of Mala s 6 which corresponds to Asp67 of Cyp B and Asp93 of CyP C is mutated to Gly59 in the sequence of CyP A. Lys74 of Mala s 6 corresponds to Lys84 in CyP B and Lys76 in CyP A, but is mutated to Val110 in CyP C. Further, Val115 of Mala s 6, which corresponds to Val125 in CyP B is mutated in CyP A and CyP C to Ala117 and Thr151, respectively. These mutations (yellow) reduce the size of the putative cross-reactive B cell epitopes, and therefore, may explain the reduced IgE-binding capacity of CyP A and CyP C compared with CyP B as seen in inhibition ELISA experiments.

The Structural Basis of Cross-Reactivity

Recently, the 3D structures of many allergens have been solved [37], allowing detailed comparisons between homologous structures. With respect to cross-reactivity to self antigens, the most interesting structures are those allowing a pair-wise comparison between the environmental and the corresponding human protein. MnSOD [6, 9, 13], CyP [8, 11] and thioredoxin [12] belong to this class, and the most exciting example is, perhaps, CyP. CyPs constitute a family of cytosolic proteins which play a pivotal role in protein folding through enzymatic catalysis of the peptidyl-prolyl *cis-trans* isomerization reaction [38]. The primary structure of this important enzyme is highly conserved among phylogenetically distant species, indicating the involvement of CyP in basic cellular functions [39]. CyP was first reported to be an allergen (Psi c 2) isolated from the basidiomycete *Psilocybe cubensis* [40], and later, from phage surface-displayed libraries of *Aspergillus fumigatus* (Asp f 11, Asp f 27) [11, 14], *Candida albicans* [8] and *Malassezia sympodialis* (Mala s 6) [8, 41]. CyP as allergen is not limited to the fungal kingdom but is also described as an allergen from various pollens [42] and foods [43].

The crystal structure of *M. symodialis* CyP (Mala s 6) has now been solved at 1.50 Å resolution [11]. Together with the solved structures of the homologous human CyPs, the new structure allows an exact comparison of the spatial orientation of conserved amino acids between self/non-self antigens. On the structural level, Mala s 6 and human CyPs superpose well with a root mean square deviation of 1.05 Å for all Cα atoms of the protein backbone. Because Mala s 6 and human CyPs show cross-reactivity in Western blot analyses [8], ELISA and skin challenges [11], they must share common IgE-binding epitopes. Obviously, only those residues that are identical in pairs of allergens and at least partly exposed to the solvent can contribute to the binding of cross-reactive IgE antibodies in native proteins. Thus, solvent-accessible residues conserved in both proteins are potentially involved in IgE-mediated cross-reactivity. Although the sequence identity between Mala s 6, human CyP A, CyP B and CyP C reach values of 71, 63 and 60% at primary structure level, respectively, only a very limited number of identical amino acids are exposed to the solvent in correctly folded protein pairs (fig. 1). The conserved surface-exposed residues are scattered over the whole sequence and are thus likely to define the discontinuous structures found in B cell epitopes. In the 3D models of the correctly folded proteins, these amino acids are clustered over the surfaces forming patches covering solvent-accessible surface areas which involve 15–22 amino acid residues on different surface loops occupying a buried surface compatible with the structure of a B cell epitope [11].

Binding of serum IgE to CyPs was determined by ELISA, and cross-reactivity was demonstrated in competitive inhibition ELISA (fig. 2). However, the observed cross-reactivity between fungal and human CyPs, as deduced from ELISA inhibition experiments using single patients' sera, ranges between 60 and 30% which is not compatible with a complete cross-reactivity. Interestingly, the strongest inhibition was obtained with human CyP B which shares the highest number of identical amino acids exposed to the solvent with Mala s 6, followed by human CyP A and human CyP C, as shown in figure 1. This might indicate that the cross-reactive epitopes bind IgE with a lower affinity depending on the number of

shared identical amino acids which determine the number of possible contacts that can be established between the allergen and the complementary determining regions of the antibodies. However, this approach is not suitable to answer the question whether the partial cross-reactivity observed between Mala s 6 and human CyPs is due to differences in affinity or to the lack of additional epitopes which could be present in the sensitizing allergen.

Clinical Relevance of Self Antigens

Autoreactivity to human proteins has been postulated as a pathogenic factor in atopic eczema (AE) based on the detection of IgE antibodies directed against various proteins in vivo [44]. There is no doubt that a variety of human proteins can induce strong B- and T-cell-mediated responses in peripheral blood mononuclear cells of patients suffering from chronic inflammatory allergic diseases [6–12]. Intradermal and skin prick tests demonstrate that self antigens are able to induce IgE cross-linking on the surface of effector cells in vivo. However, a positive dermal challenge test does not necessarily correlate with clinical symptoms since up to 40% of the individuals sensitized to environmental allergens are asymptomatic [45]. Therefore, skin test challenges with self antigens are useful to demonstrate cross-reactivity with homologous recombinant allergens or direct sensitization to the self antigen, but not for the demonstration of the clinical relevance. Although it makes sense to assume that self antigen-induced mediator release in vivo could have clinical consequences at least for a subset of the patients, we presently do not have any suitable test to unequivocally demonstrate an involvement of self antigens in the pathogenesis of allergic diseases.

Probably the most reliable indication for a role of self antigens in the pathogenesis of allergic diseases comes from atopy patch tests performed with human MnSOD in patients suffering from AE [9, 46]. This study showed that human MnSOD – a stress-inducible enzyme – is able to induce eczematous reactions on unaffected skin areas of AE patients sensitized to *M. sympodialis*. In concordance with the presence of MnSOD-specific IgE, the human enzyme also elicited positive skin prick test reactions and T cell proliferation in all ELISA-positive patients. Interestingly, reactivity against human MnSOD strongly correlated with the severity of the disease, further corroborating the postulated pathogenic role of self-reactivity in the exacerbation of AE [9]. A further indication that such reactions might contribute to the pathogenesis of AE derives from the observation that human MnSOD is upregulated in eczematous areas as shown by immunohistochemistry, most likely as a consequence of mechanical stress due to scratching. Interestingly, also CyPs and thioredoxins represent stress-inducible enzymes which are upregulated as a consequence of cellular stress. Oxidative stress could, through upregulation of nuclear factor-κB expression, induce interleukin-4 production [47] and, as a consequence thereof, accentuate Th2 responses to allergens and homologous self antigens.

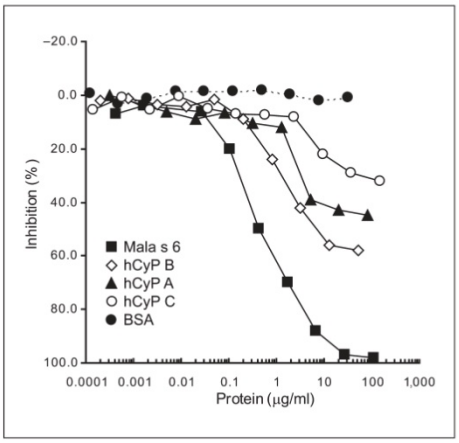

Fig. 2. Inhibition of IgE binding to solid phase-coated Mala s 6, with Mala s 6, homologous human CyPs and BSA as a negative control. Serum of a Mala s 6 sensitized patient was preincubated with increasing concentrations of Mala s 6, human CyP B, CyP A, CyP C and BSA as negative control. After transfer of the preincubated serum to Mala s 6 coated wells, the residual IgE binding was detected by ELISA. Apparently, all human CyPs are able to inhibit the binding of IgE to Mala s 6, indicating that Mala s 6 and the homologous human proteins share common cross-reactive IgE-binding epitopes. The reduced capacity to inhibit the IgE binding to Mala s 6 of CyP A and CyP C compared with CyP B can be explained on the structural level of these proteins.

Concluding Remarks

Evidence is increasing that self antigens are a contributing factor to the exacerbation of long-standing, inflammatory atopic diseases. Structural data provide information allowing a rational explanation of cross-reactivity

between environmental allergens and structurally related self antigens. There is no doubt that structural features which are shared between self antigens such as MnSOD, CyPs and thioredoxin and homologous environmental (pan)-allergens are responsible for the common IgE-binding capacity observed in Western blot analyses, ELISA and competitive inhibition ELISA, as well as for their ability to elicit positive skin or atopy patch tests. Cross-reactivity between these molecules is never complete, indicating that either environmental allergens elicit, beside cross-reactive, additional IgE-binding epitopes, or that the B cell epitopes displayed on the surface of self antigens have a reduced IgE-binding affinity. Either reason or their combination could be sufficient to explain the reduced IgE-binding capability of self antigens compared with their homologous environmental allergens. In contrast to the cross-reactivity between human and homologous environmental allergens which was studied in deep detail, not much is known about the autoreactivity of self antigens without sequence homology to environmental allergens. It is still not clear if they are able to directly induce a B cell switch towards IgE production, or if the observed IgE-binding capability is due to cross-reactivity with not yet identified environmental allergen structures. However, clear evidences showing that autoreactivity could be a pathogenic factor for severe chronic allergic diseases are still lacking, and the only indication that this could be the case is limited to the observation that the levels of IgE autoantibodies in AE patients correlate to the severity of the disease.

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8.6 Conclusion and Outlook

Within the present thesis 140 new self-antigens potentially associated with AE were identified. Five randomly selected proteins together with the known IgE-binding self-antigens cyclophilin B and thioredoxin were shown to bind IgE in sera from AE-patients, to induce mediator release from basophils, and to stimulate the proliferation of T cells from sensitized patients.

Among the repertoire of IgE-binding self-antigens several phylogenetically highly conserved proteins such as actin-α and tubulin-α are present. These self-antigens are supposed to cross-react with homologous environmental allergens due to molecular mimicry. Whether molecular mimicry is the only basis of IgE-mediated autoreactivity or whether sensitization to self-antigens can arise as a direct consequence of inflammatory processes remains to be elucidated.

Except HLA-DR-α, all characterized IgE-binding self-antigens are intracellular proteins that are supposed to be released during inflammatory processes and thereby become accessible to the immune system. In contrast, HLA-DR-α is permanently displayed on the outer surface of antigen-presenting cells, but did not activate patients' basophils most likely due to monovalent IgE-binding. Therefore, the detailed study of HLA-DR-α and further self-antigens that are secreted or exposed on the cell surface will be the subject of interesting future studies.

Finally, a detailed analysis of patients' characteristics and IgE-mediated autoreactivity might lead to the identification of subgroups of patients that are for instance at high risk to develop further atopic diseases or suitable for SIT. Thus, IgE-mediated autoreactivity could lead to the development of new diagnostic criteria for the stratification of AE patients in order to identify underlying disease mechanism aimed to offer the patients proper advice and treatment.

8.7 Statement of contribution to publications

For the article entitled “Exploring the repertoire of IgE-binding self-antigens associated with atopic eczema” I performed all experiments (Figure 6-10) under the supervision of R. Crameri. C. Rhyner provided technical advice, N. Meyer and P. Schmid-Grendelmeier recruited the patients enrolled in my studies.

For the short communication article entitled “The IgE-binding self-antigens tubulin-α and HLA-DR-α are overexpressed in the lesional skin of atopic eczema patients” I performed all experiments (Figure 11) with the technical support of C. Johansson and C. Rhyner under the supervision of A. Scheynius and R. Crameri.

The review article “Immunoglobulin-E-Mediated Reactivity to Self-Antigens: A Controversial Issue” was written by myself under guidance of R. Crameri. A. Glaser, M. Vilhelmsson, and C. Rhyner provided experimental results.

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10 Curriculum Vitae

Name	Zeller
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Place of birth	Villingen, Germany
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Education

10/2005 – 04/2009	*Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland* **Doctorate (PhD) in Allergology** Title of the project: *Probing the IgE-binding repertoire of self-antigens in atopic eczema*
04/2008 – 06/2008	*Karolinska Institute, Stockholm, Sweden* **Research fellowship**
10/2000 – 08/2005	*University of Konstanz, Konstanz, Germany* **Diploma in biology (Dipl. Biol.)** Special focus on biochemical pharmacology, immunology, microbiology, and molecular toxicology Title of the diploma thesis: *Establishment of methods for the activation of Chlamydophila pneumonia-specific T cells*
06/2004 – 08/2004	*Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland* **Internship**
03/2004 – 05/2004	*Max Planck Institute for Medical Research, Heidelberg, Germany* **Internship**
06/2000	*Gymnasium am Romäusring, Villingen, Germany* **Abitur**

Awards and Honors

Prizes for excellent scientific presentations at the XXVII Congress of the European Academy of Allergology and Clinical Immunology (EAACI) and at the Meeting of PhD-Students in Grisons (Young Scientists in Contest)

Fellowships from the European Molecular Biology Organization (EMBO) and from the European Academy of Allergology and Clinical Immunology (EAACI) to finance a research stay at the Karolinska Institute in Stockholm

Prize of the University of Konstanz for excellent Diploma-studies in Biology

Attended courses during PhD

02/2009	7th EAACI-GA^{2}LEN Davos Meeting, Winter School, **Basic Immunology Research in Allergy and Asthma**, Davos, Switzerland
11/2008	**The successful start of a professional career**, Life Science Zürich Graduate School, University of Zürich, Switzerland
11/2007	**Scientific writing**, Life Science Zürich Graduate School, University of Zürich, Switzerland
06/2007	**Study design and data analysis**, Life Science Zürich Graduate School, University of Zürich, Switzerland

Presentations given in SIAF

Progress reports	**Journal Clubs**	**SIAF Science day**
June 06, 2006	April 25, 2006	December 13, 2007
November 28, 2006	October 03, 2006	December 17, 2008
May 29, 2007	June 21, 2007	
November 27, 2007	October 02, 2007	
September 30, 2008	November 17, 2008	
March 10, 2009		

Meeting of "Promotionskomitee"

October 11, 2006
October 12, 2007
October 13, 2008

Attendance of Congresses

Oral presenations

7th EAACI-GA^{2}LEN Davos Meeting, Winter School, Basic Immunology Research in Allergy and Asthma, February 2009, Davos, Switzerland

6th EAACI-GA^{2}LEN Davos Meeting, Basic Immunology Research in Skin Allergy and Immunotherapy, February 2008, Pichl / Schladming, Austria

Klinisch-experimentelle Konferenz, December 2007, SIAF, Davos und USZ, Zürich, Switzerland

Poster presentations

World Immune Regulation Meeting III, Special focus on regulatory and effector mechanisms, March 2009, Davos, Switzerland

Young Scientist in Contest, August 2008, Davos, Switzerland

XXVII EAACI Congress, June 2008, Barcelona, Spain

World Immune Regulation Meeting II, Special focus on regulatory cells and Th17 cells, March 2008, Davos, Switzerland

World Immune Regulation Meeting I, Special focus on regulatory cells, April 2007, Davos, Switzerland

XIX. Meeting of the Swiss Immunology Ph.D. Students, March 2007, Schloss Wolfsberg, Ermatingen, Switzerland

5th EAACI-GA^{2}LEN Davos Meeting, Basic Immunology Research in Allergy and Clinical Immunology, February 2007, Davos, Switzerland

List of Publications

Original Articles

1. **Zeller, S.**, C. Johansson, C. Rhyner, A. Scheynius, and R. Crameri. The IgE-binding self-antigens tubulin-alpha and HLA-DR-alpha are overexpressed in the lesional skin of atopic eczema patients. *Allergy* submitted.

2. **Zeller, S.**, C. Rhyner, N. Meyer, P. Schmid-Grendelmeier, C.A. Akdis, and R. Crameri. Exploring the repertoire of IgE-binding self-antigens associated with atopic eczema. *J Allergy Clin Immunol* accepted for publication.

3. Bunk, S., H. Schaffert, B. Schmid, C. Goletz, **S. Zeller**, F. Kern, J. Rupp, and C. Hermann. Presence of Chlamydia pneumoniae-specific IFN-gamma-and IL-2-producing memory CD4+ T cells correlates with lower probability of persistent infection. *Eur J Immunol* in revision.

4. Glaser, A.G., G. Menz, A.I. Kirsch, **S. Zeller**, R. Crameri, and C. Rhyner. 2008. Auto- and cross-reactivity to thioredoxin allergens in allergic bronchopulmonary aspergillosis. *Allergy* 63:1617-1623.

5. Glaser, A.G., A.I. Kirsch, **S. Zeller**, G. Menz, C. Rhyner, and R. Crameri. 2008. Molecular and Immunological characterization of Asp f 34, a novel major cell wall allergen of Aspergillus fumigatus. *Allergy* in press.

6. Limacher, A., A.G. Glaser, C. Meier, P. Schmid-Grendelmeier, **S. Zeller**, L. Scapozza, and R. Crameri. 2007. Cross-reactivity and 1.4-A crystal structure of Malassezia sympodialis thioredoxin (Mala s 13), a member of a new pan-allergen family. *J Immunol* 178:389-396.

7. Rauter, C., M. Mueller, I. Diterich, **S. Zeller**, D. Hassler, T. Meergans, and T. Hartung. 2005. Critical evaluation of urine-based PCR assay for diagnosis of Lyme borreliosis. *Clin Diagn Lab Immunol* 12:910-917.

Review Articles

1. Crameri, R., **S. Zeller**, A.G. Glaser, M. Vilhelmsson, and C. Rhyner. 2009. Cross-reactivity among fungal allergens: a clinically relevant phenomenon? *Mycoses* 52:99-106

2. **Zeller, S.**, A.G. Glaser, M. Vilhelmsson, C. Rhyner, and R. Crameri. 2008. Immunoglobulin-E-mediated reactivity to self antigens: a controversial issue. *Int Arch Allergy Immunol* 145:87-93.

3. **Zeller, S.**, A.G. Glaser, C. Rhyner, and R. Crameri. 2007. Kreuz-und Autoreaktivität von Allergenmolekülen aus struktureller Sicht. *Allergologie* 30:269-276.

4. Crameri, R., A. Limacher, A.G. Glaser, **S. Zeller**, C. Rhyner, and M. Weichel. 2007. The Molecular Basis of IgE-Mediated Autoreactivity. *Allergy and Clin Immunol Int: J World Allergy Org* Supplement 3.

5. Crameri, R., A. Limacher, M. Weichel, A.G. Glaser, **S. Zeller**, and C. Rhyner. 2006. Structural aspects and clinical relevance of Aspergillus fumigatus antigens/allergens. *Med Mycol* 44 Suppl:261-267.

Book chapters

1. Schmid-Grendelmeier, P., **S. Zeller**, and R. Crameri. Fungi in Atopic Dermatitis. In Atopic Dermatitis. T. Bieber, and D. Leung, editors. in press.

Abstracts published in peer reviewed journals

1. Rhyner, C., A.G. Glaser, A.I. Kirsch, **S. Zeller**, G. Menz, and R. Crameri. 2009. Identification and characterization of Asp f34 a novel major allergen of A. fumigatus. *Swiss Medical Weekly* 139:8S-8S.

2. **Zeller, S.**, A. Glaser, C. Rhyner, and R. Crameri. 2008. A broad spectrum of IgE-binding self-antigens in atopic eczema. In 27th Congress of the European-Academy-of-Allergology-and-Clinical-Immunology. Barcelona, SPAIN. 1499.

3. Rhyner, C., A.G. Glaser, A. Limacher, **S. Zeller**, and R. Crameri. 2007. The structural basis of IgE-mediated autoreactivity in atopic eczema. *Swiss Medical Weekly* 137:9S-9S.